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THE THRESHOLD OF ANGULAR ACCELERATION PERCEPTION

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(*Received* 4 *November* 1946)

The threshold for perception of angular acceleration is by no means a well-established quantity, although many investigators have attempted its determination by various methods. Mach (1875) described a method by which he determined the threshold for the sensation of rotation with a torsion swing. He found a threshold value of $2^\circ/\text{sec.}^2$, as did Mulder (1908) and van Wulfften Palthe (1922). Tönnies (1932) and Woletz (1932), using an electrically driven rotating chair, also found $2^\circ/\text{sec.}^2$; Arslan (1934), however, arrived at only $0{\cdot}5\text{–}1^\circ/\text{sec.}^2$

Buys (1940) found that a minimum acceleration of $1^\circ/\text{sec.}^2$ was required to cause nystagmus in men.

Buys & Rijlant (1939) found, for a large number of test-animals, that an average of $0{\cdot}8^\circ/\text{sec.}^2$ was required to cause nystagmus of the head. Mowrer (1935) reported that $0{\cdot}43\text{–}1{\cdot}56^\circ/\text{sec.}^2$ was required to cause nystagmus of the head in the pigeon. Ter Braak (1936), observing the minimum ocular deviations of rabbits photographically, found the much lower threshold of $0{\cdot}1^\circ/\text{sec.}^2$

Mulder (1908) was the first to point out that the product of the time and the acceleration required to reach the threshold of rotational sensation is constant. Thus, for reaching the threshold, the required acceleration is the greater, the shorter the time of its action.

METHODS AND RESULTS

The minimum value for the perception of angular acceleration has been determined by various methods. We shall describe these methods in some detail, because it is necessary to check the various mechanical formulae which have been deduced theoretically (van Egmond, Jongkees & Groen, 1943).

Experiments with the rotating chair

Using the rotating chair we found for the minimum acceleration that produces a sensation of rotation after an infinitely long time (in practice this is about 30 sec.) an average value of 0·8°/sec.^{2} for thirty subjects, the extreme values observed being 0·28 and 2·0°/sec.^{2}

One reason why we preferred to determine the threshold by means of the sensation aroused instead of depending upon the deviation of the eye, as recommended by Ter Braak (1936), was the fact that nothing was known about the purely mechanical forces which might cause a rotation of the eye. Such a purely mechanical influence was found for the rolling of the eyes by Kompanejetz (1924). As the inertia of the eye rather than the reaction of the labyrinth might be the measured quantity, it seemed possible that values obtained in that way might be appreciably too low. Owing to circumstances caused by the war we were not able to investigate this problem experimentally until after our own experiments were finished. We reached the following conclusions:

The eye is approximately a sphere that can rotate about its centre. If an angular acceleration is given to the head, the eye tends to lag behind owing to its inertia, but to move with the head owing to the friction between eyeball and orbit, and because of the action of the eye muscles. This analysis is strictly mechanical and neglects the alterations of muscle tone depending upon the activity of the labyrinth. Thus, if the eye is to be displaced, the angular acceleration α must be such that the inertial torque just exceeds the frictional torque W. If θ is the moment of inertia of the eye we have

$$W = \theta\alpha.$$

We have investigated what force is just capable of starting a rotation of the eye by pushing a hair tangentially against a scarified spot of the cocainized sclera of a rabbit eye and measuring the deviation of the position of a little egg-white, marked with a cross, stuck to the cornea. With a hair that could exert a pressure of 0·15 g., as measured on a balance, a just visible deviation was obtained. This corresponds to a torque

$$W = 0{\cdot}150 \times 981 \times 0{\cdot}85 \text{ dyne/cm.},$$

the radius of the rabbit eye being approximately 0·85 cm. The moment of inertia

$$\theta \approx 0{\cdot}24\, mr^2 \approx 0{\cdot}74.$$

The angular acceleration is, therefore,

$$\alpha = \frac{0{\cdot}150 \times 981 \times 0{\cdot}85}{0{\cdot}74} = 170 \text{ rad./sec.}^2 = 10{,}000^\circ/\text{sec.}^2 \text{ approx.}$$

Hence, the angular acceleration which is capable of causing a visible deviation of the eye, apart from any interaction with the semicircular canals, must be at least 10,000°/sec.^{2} This value is much too high to be of any influence on the determination of the threshold for the effect of angular accelerations on the eye. The values given by Ter Braak may, therefore, be trusted.

Experiments with the torsion swing

For ourselves a series of determinations yielded the values of Tables 1 and 2. The average threshold value for J was 0·25°/sec.^{2}, and for G 0·18°/sec.^{2} The corresponding values given by the rotating-chair method were 0·45 and 0·28°/sec.^{2} respectively.

TABLE 1. Torsion-swing values for the angular acceleration threshold of subject J

T (sec.)	11·4	10·9	11·7	6·34	6·10	5·30
A (°)	3·33	3·80	2·60	2·45	2·15	1·65
Threshold (°/sec.^{2})	0·22	0·26	0·17	0·32	0·27	0·23

TABLE 2. Torsion-swing values for the angular acceleration threshold of subject G

T (sec.)	10·65	10·7	10·5	5·88	5·77	4·95
A (°)	2·20	3·50	3·00	1·65	1·02	0·80
Threshold (°/sec.^{2})	0·16	0·25	0·22	0·21	0·13	0·12

As was expected, the values derived with the torsion swing are lower than those derived from experiments with the rotating chair, the difference being about 30%. Moreover, Mach's torsion swing data (see discussion) are in good agreement with the average value of 0·8°/sec.^{2} of our experiments with the rotating chair, his figure of about 0·55°/sec.^{2} being 30% less, in accordance with expectation.

Indirect measurement of the threshold

There are, however, other methods for the determination of the threshold of angular acceleration. The constancy of the effect of the product of acceleration and time, the so-called αt law of Mulder, theoretically permits the measurement of the threshold value for acceleration by determining the minimum perceptible momentum.

Analysis of the processes in the canals leads to the conclusion (to be discussed in detail elsewhere) that the deflexion x of the cupola is

$$x = \alpha t \frac{\theta}{P},$$

where x = angular displacement of the endolymph in radians, α = angular acceleration in rad./sec.^{2}, t = time in sec., θ = moment of inertia, P = frictional torque at unit angular velocity, and

$$x_{\text{min.}} = C_{\text{min.}} \frac{\theta}{P} z,$$

where C = momentum in rad./sec.^{2} and z = correction factor.

Equating x,

$$\alpha t = C_{\text{min.}}\, z.$$

From the minimum momentum, that is the minimum angular velocity at which a sensation of rotation is caused by sudden deceleration, it should be possible to deduce the minimum perceptible angular acceleration. In practice, however, difficulties arise, because the change of momentum continues to be detected by tactile and pressure receptors when the sensation of rotation has ceased. Inexperienced observers will therefore find values for the minimum momentum which are too low. On the other hand, in the case of experiments with the rotating chair, the value of αt obtained will generally be too high, because it will include reaction time.

A survey of our results may elucidate these questions.

Subject	αt	$C_{\text{min.}}$
J	4·5	4·1
G	1·5	1·3
S	3·6	2·0
R	4·7	1·2
I	4·5	2·5
E	14·0	4·8
P	3·0	3·0
L	3·4	1·0
Ro	2·3	1·5
K	3·1	2·0

The results agree very well for the most experienced subjects J, G and P, but for all other subjects differences are evident, the αt product being always larger than the minimum momentum. The factor z of the formula is a correction factor of the order of magnitude of unity and therefore does not influence the result.

Another more practicable method consists in giving a subject a momentum C rad./sec. and observing the duration, t sec., of the after-sensation for various values of C,

$$t=f(C).$$

From the equations of motion of the endolymph (see the preliminary communication of van Egmond *et al.* (1943); we hope to discuss this matter in more detail soon) it follows that

$$x_{\text{min.}}=\frac{C\theta}{P}\,\text{exp.}\left[-\frac{D}{P}t_{\text{min.}}\right],$$

and

$$t_{\text{min.}}=\frac{P}{D}\ln\frac{C\theta}{Px_{\text{min.}}},$$

where D = directional force per unit angle. Hence the relaxation time after the change of momentum also depends on the threshold for perception of angular acceleration.

If the time of after-sensation is plotted as a function of the magnitude of the momentum, as was done in Fig. 1, the threshold may be derived from the figure. In this figure the full-drawn line represents the theoretical relation between the time of after-sensation and the change of momentum for the subject with perception threshold of $0{\cdot}41°/\text{sec.}^2$ according to the above formula, this value being the threshold of one of the subjects tested. The results obtained by this method were in good agreement with those obtained in other ways. Their magnitudes lay between those obtained with the torsion swing and those found with the rotating chair. Here, too, the criterion for the determination is a different one, namely, the time for which it is possible to observe a gradually vanishing sensation of rotation. As with the rotating chair the test person is subjected to an angular acceleration and, therefore, the signal that the minimum point is reached will always be given a little too late. The result of this error in these experiments is that the threshold found is somewhat too low, whereas in the case of the experiments with direct application of angular acceleration this is not so.

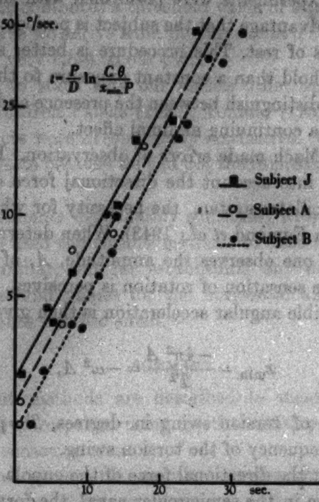

Fig. 1. The duration of the after-sensation after receiving a momentum is plotted as a function of the magnitude of the momentum. Abscissa, time in sec.; ordinate, magnitude of the momentum on a logarithmic scale in degrees/sec. The full-drawn curve represents the duration of the after-sensation according to the formula

$$t=\frac{P}{D}\ln\frac{C\theta}{Px_{\text{min.}}},$$

for a person with a threshold of 0·41°/sec.^{2} The lines found experimentally for the subjects A and B indicate that their thresholds lie at 0·8 and 0·6 times this value, hence for A at 0·33°/sec.^{2} and for B at 0·25°/sec.^{2} For the plotting of the formula for t we made use of the numerical values found by van Egmond *et al.* (1943): $P/D=8{\cdot}3$ and $\theta/P=0{\cdot}12$.

DISCUSSION

Thirty subjects were tested according to the three methods described, and a threshold value for the perception of angular acceleration on the basis of the rotational sensation was obtained. The method with the torsion swing appeared to be the most sensitive, whereas the method with the rotating chair and small enduring accelerations was less sensitive. The values found were of the order of magnitude of 0·5°/sec.^{2} The average value was found somewhat higher, mainly because a few persons had an appreciably higher threshold. The highest value found was 2·0°/sec.^{2}, the next one was 1·4°/sec.^{2} Owing to these two abnormally high values our average value is a little high.

The first investigators obtained higher values than their successors. This is remarkable in the case of Mach (1875) who used a method that leads one to expect lower values than those obtained with the direct method on the rotating chair. His experiments were performed with the torsion swing and this method has the advantage that the subject is periodically subjected to the stimulus with periods of rest. This procedure is better suited for the determination of the threshold than a constant exposure to the minimal stimulus, because it is easier to distinguish between the presence and absence of an effect than to be certain of a continuing minimal effect.

It is unlikely that Mach made errors of observation. It appears, however, that he did not take into account the directional force of the cupola in the endolymph-cupola oscillator-system, the necessity for which was pointed out by us previously (van Egmond *et al.*, 1943). When determining the threshold on the torsion swing one observes the amplitude, A, of the swing with the period T at which the sensation of rotation is perceived. According to Mach the minimum perceptible angular acceleration is then given by

$$x_{\text{min.}} = \frac{-4\pi^2 A}{T^2} = -\omega^2 A,$$

where A = amplitude of torsion swing in degrees, T = period of oscillation in sec., ω = circular frequency of the torsion swing.

Taking into account the directional force of the cupola and the damping of the endolymph motion in the semicircular canal, the formula should be used in the form

$$x_{\text{min.}} = \frac{A\omega\theta}{P}\cos\omega t,$$

in which permissible approximations are applied to the expansion of the formula for the endolymph displacement x with a forced oscillation with circular frequency ω, the resonance circular frequency of the endolymph cupola system being ω_0

$$x = -\frac{\omega^2 A \sin(\omega t - \phi)}{\sqrt{[(\omega_0^2 - \omega^2)^2 + (P^2/\theta^2)\,\omega^2]}},$$

where ϕ = auxiliary angle defined by $\phi = \dfrac{\omega P}{(\omega_0^2 - \omega^2)\,\theta}$, and ω_0 = circular frequency of the cupola endolymph system.

When the amplitude A decreases, the threshold is reached. If $\omega = \omega_0$ we have

$$x = -\frac{\omega A\theta}{P}\cos\omega t.$$

Because the term $\left(\dfrac{P\omega}{\theta}\right)^2$ is large compared with $(\omega_0^2 - \omega^2)^2$ this formula is a good approximation also for the case $\omega_0 \neq \omega$.

Mach finds for himself and for his student Dvořak

Mach: $T = 14$ sec., $A = 10°$.
Dvořak: $T = 16$ sec., $A = 13°$.

From these results he computes a threshold value of $2°/\text{sec.}^2$ According to the method outlined above we derive from the same data $0{\cdot}57°/\text{sec.}^2$ and $0{\cdot}52°/\text{sec.}^2$ respectively, values very close to our own.

We are not able to state why our results differ appreciably from those obtained by van Wulfften Palthe (1922), Tönnies (1932) and Woletz (1932). Possibly their subjects were exceptionally insensitive. Our observations were made on young subjects because we had got the impression that older subjects are less sensitive.

Though our experiments were mainly concerned with the horizontal pair of canals, we have also used the various methods to examine the vertical canals. The results were similar, but for establishing any difference between the average of the threshold values for the horizontal and vertical canals the number of experiments was too small.

SUMMARY

1. Three different methods are described to measure the threshold of angular acceleration perception, using (*a*) the rotating chair, (*b*) the torsion swing, (*c*) the after-sensation time.

2. The minimum perceptible angular acceleration for man is about $0{\cdot}5°/\text{sec.}^2$

3. The experiments of Mach (1875) yield the same result, if complete mechanical data are used. Mach's calculation, leading to a threshold value of $2°/\text{sec.}^2$, neglects the directional force and damping of the cupola.

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RENAL EXCRETION OF SODIUM AND POTASSIUM IN RATS

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Renal excretion of potassium has been investigated in man (Griffon, 1936; Cutler, Power & Wilder, 1938; Hall & Langley, 1940), in the dog (Winkler & Smith, 1942) and in the cat (Wirz, 1945), but opinions as to its mechanism are divergent. It is not clear from these papers whether the amount of potassium excreted is related to the rate of glomerular filtration. It was, therefore, decided to investigate the mechanism of potassium excretion in rats and to compare it with that of chloride and of sodium.

METHODS

Experimental animals. Male adult albino rats were used. The animals, before being used, were on a standard diet for several weeks.

Experimental procedures. Inulin was injected subcutaneously and 5% of the body weight of tap water was given by stomach tube. The routine procedure for determining the clearances conformed with that described previously (Dicker & Heller, 1945).

Analytical methods. Inulin in plasma and in urine was determined by the method of Smith, Goldring & Chasis (1938). Chloride in plasma was determined by Whitehorn's (1921) method, and in urine by that of Volhard (1878). Sodium in plasma and in urine was determined by the method of McCance & Shipp (1931), and potassium in plasma and in urine by Kramer & Tisdall's method as modified by McCance & Shipp (1933); the empirical factor used in the calculation being that proposed by McCance & Shipp, i.e. 0·12. The inulin preparation used was Inulin (Kerfoot & Co.).

Method of calculation. The following formula was used to calculate the tubular reabsorption rate of electrolytes (T_E):

$$C_{In} \times E_p - U \times E_u = T,$$

where C_{In} = inulin clearance/min./100 g. = glomerular filtration rate (G.F.R.), E_p = concentration of the ion E per 100 c.c. plasma, U = urinary volume/min./100 g. body weight and E_u = concentration of the ion E per 100 c.c. urine. To allow for possible variations of the glomerular filtration rate, T was expressed as percentage of the amount of ion E filtered thus:

$$T_E = \frac{T}{C_{In} \times E_p} \times 100.$$

Statistical treatment of results. A 'small sample' method was used throughout for the estimation of the significance of means (Fisher, 1944). As the population of the samples was small, the standard deviation was multiplied by $\sqrt{(n/n-1)}$ (Bradford Hill, 1942). Correlation coefficients (r) were calculated according to Mainland (1938). The values for the probability (P) of r and of t were obtained from the Fisher & Yates (1943) tables.

RESULTS

Renal excretion of chloride, sodium and potassium during a water diuresis. It has been shown (Dicker & Heller, 1945) that the G.F.R. of rats does not change significantly during a water diuresis. The mean value for G.F.R. in the present series amounted to $0{\cdot}39 \pm 0{\cdot}024$ c.c./100 g. rat/min. (S.E. of mean of twelve observations). The mean values for the concentration of chloride, sodium and potassium in the plasma were:

$$274{\cdot}4 \pm 8{\cdot}4\ (9),\ 283{\cdot}5 \pm 13{\cdot}0\ (9)\ \text{and}\ 24{\cdot}2 \pm 0{\cdot}3\ (12)\ \text{mg./100 c.c.},$$

respectively. The mean amount of the three ions filtered through the glomeruli was therefore: 1·08 mg./100 g./min. for chloride, 1·12 mg./100 g./min. for sodium and 0·09 mg./100 g./min. for potassium. It will be seen from Fig. 1 that the reabsorption of chloride and that of sodium, expressed as percentages of the amount of chloride and of sodium filtered, remained unchanged with variations of urine flow ranging from 0·0046 to 0·0650 c.c./100 g./min.; in other words the rates of chloride and of sodium reabsorption (T_{Cl} and T_{Na}) remained practically constant at the very low and at the very high rates of urine formation encountered in the experiments. They were independent of the amount of chloride and sodium filtered and of the rate of water reabsorption. The reabsorption of chloride, expressed as percentage of chloride filtered (T_{Cl}) amounted to $96{\cdot}0 \pm 0{\cdot}9\%$ (9) and that of sodium (T_{Na}) to $97{\cdot}4 \pm 0{\cdot}6\%$ (9) (Table 1). It will be noted that the rate of tubular reabsorption of chloride was significantly different from that of sodium ($t = 3{\cdot}070$, $P < 0{\cdot}02$).

The rate of potassium reabsorption was quite different from that of sodium and of chloride. It was high at low rates of urine flow and low at high rates of urine formation (Fig. 1, A, I). The mean rate of potassium reabsorption, expressed as percentage of the amount of potassium filtered (T_K), was $68{\cdot}0 \pm 3{\cdot}27\%$; it was found to be related (*a*) to the amount of potassium filtered, $r = -0{\cdot}940$, $P < 0{\cdot}001$ (coefficient of regression, $b = -5{\cdot}31$); (*b*) to the rate of water reabsorption (T_W), $r = +0{\cdot}757$, $P < 0{\cdot}01$; and (*c*) to the urine flow, $r = -0{\cdot}887$, $P < 0{\cdot}001$ (Table 1).

It is thus clear that in rats during a normal water diuresis, the rates of chloride and of sodium reabsorption are independent of the rate of water reabsorption and the rate of urine flow, but that the rate of potassium reabsorption varies with the rate of water reabsorption and, therefore, with the urinary output.

Renal excretion of chloride, sodium and potassium after injection of potassium chloride. To determine how an increase of plasma potassium affects potassium excretion, rats were injected subcutaneously with 0·1 c.c./100 g. body weight of a 3·0% KCl solution. Inulin, chloride, sodium and potassium clearances were estimated in the usual way.

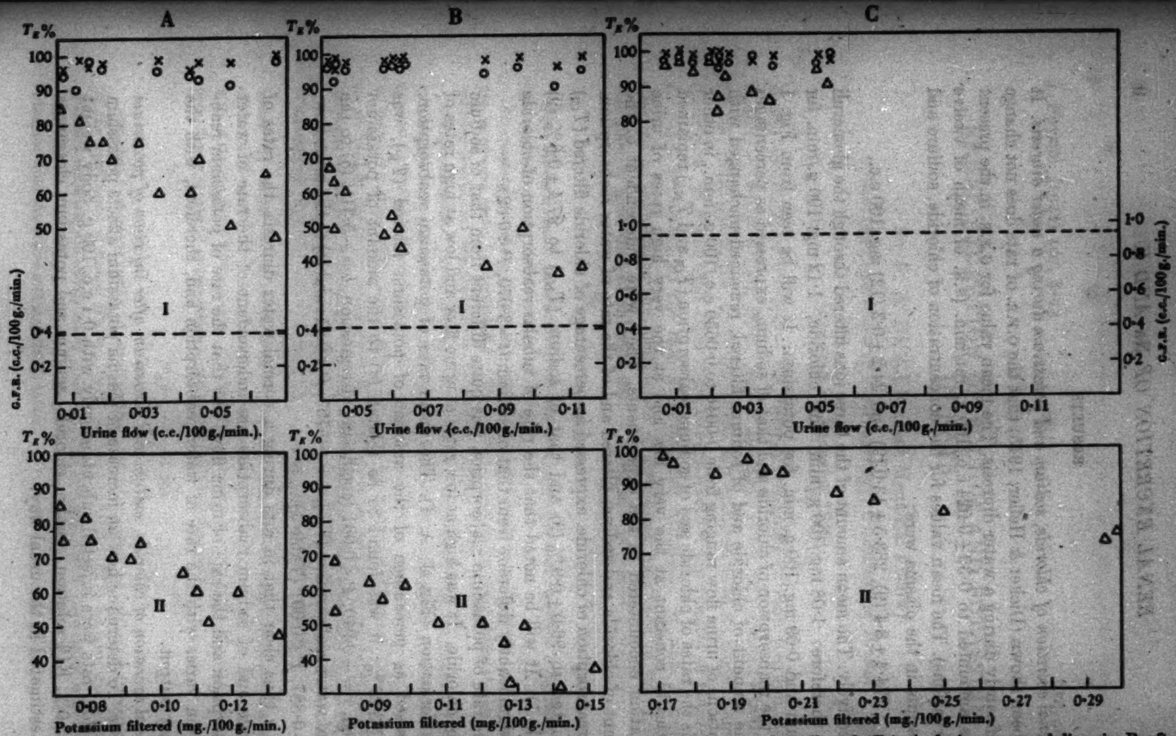


Fig. 1. Electrolyte reabsorption per min./100 g. rat expressed as percentage of the amount of electrolyte filtered (T_E): A, during a normal diuresis; B, after injection of 0·1 c.c. of a 3·0% KCl solution; C, after administration of 5·0 c.c. of an 85 mM solution of NaCl per 100 g. rat. In I, the electrolyte reabsorption (T_E) is plotted against the rate of urine flow, expressed as c.c./100 g./min. In II, potassium reabsorption (T_K) is plotted against the amount of potassium filtered, expressed as mg./100 g./min. × = chloride; o = sodium; △ = potassium. The broken line indicates the mean value obtained for the G.F.R.

TABLE 1

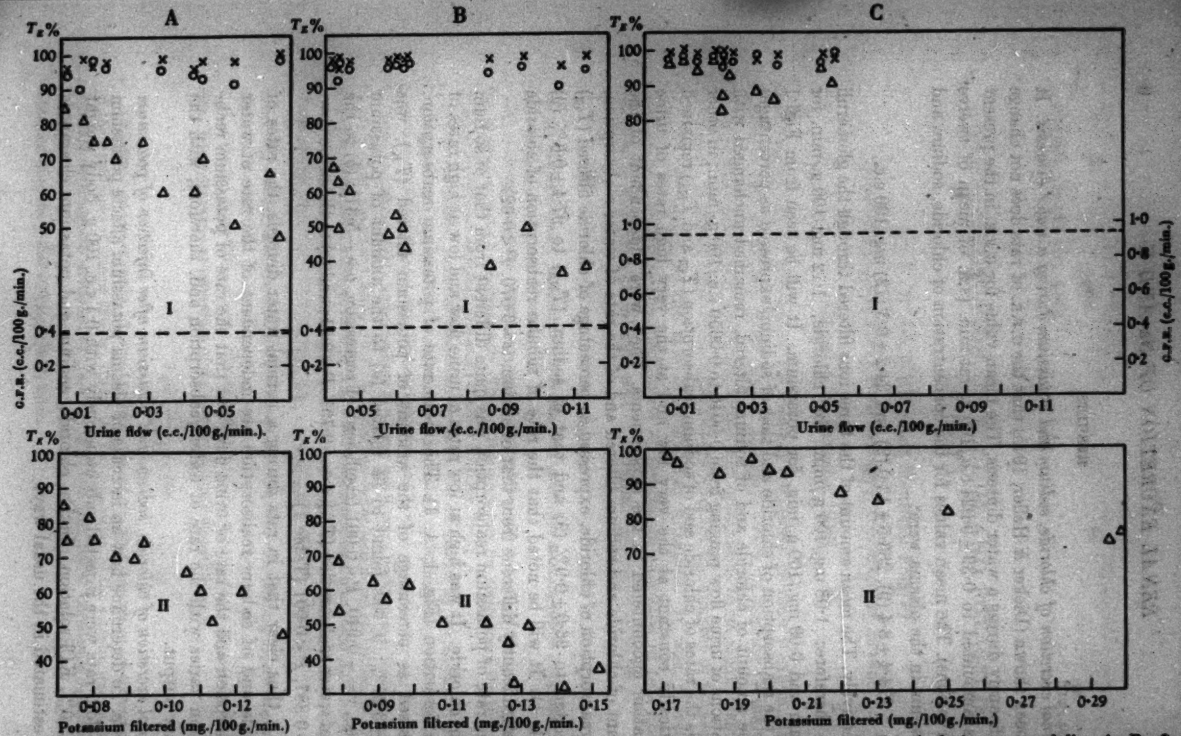
	G.F.R. (c.c./100 g./min.)	Cl filtered (mg./100 g./min.)	Na filtered (mg./100 g./min.)	K filtered (mg./100 g./min.)
Water diuresis	0·39 ±0·024 (12)	1·08 ±0·165 (9)	1·12 ±0·047 (9)	0·09 ±0·006 (12)
After injection of 0·1 c.c./100 g. of a 3·0 % KCl solution	0·41 ±0·042 (12)	1·39 ±0·282 (12)	1·21 ±0·263 (12)	0·11 ±0·007 (12)
After administration of 5·0 c.c./100 g. of 85 mM solution of NaCl	0·94 ±0·049 (11)	3·01 ±0·163 (11)	3·04 ±0·134 (11)	0·22 ±0·013 (11)

	T_{Cl} (%)	T_{Na} (%)	T_K (%)	r between T_K and T_W	r between T_K and K filtered	r between rate of urine flow and T_W
Water diuresis	96·0 ±0·96 (9)	97·4 ±0·62 (9)	68·0 ±3·27 (12)	+0·757 —	−0·940 —	−0·995 —
After injection of 0·1 c.c./100 g. of a 3·0 % KCl solution	97·4 ±0·57 (12)	98·3 ±0·28 (12)	48·4 ±3·22 (12)	+0·660 —	−0·950 —	−0·977 —
After administration of 5·0 c.c./100 g. of 85 mM solution of NaCl	97·8 ±0·32 (11)	98·6 ±0·14 (11)	91·7 ±2·51 (11)	+0·365 —	−0·955 —	−0·909 —

The figures are mean results with standard errors; r = correlation coefficient between the values indicated.

Number of experiments in brackets.

Such an injection resulted in an increase of potassium and of chloride in the plasma, and had a pronounced diuretic effect. The mean value for the G.F.R. was 0·41 ± 0·042 (12) c.c./100 g./min., a value which is comparable to that observed during a normal water diuresis. The mean plasma chloride amounted to 339·9 ± 6·42 (12) mg./100 g., and the plasma potassium was 27·2 ± 0·71 (12) mg./100 c.c., compared with 24·2 ± 0·28 in normal rats. However, the plasma sodium remained normal (293·5 ± 7·09 (12) mg./100 c.c.). The increase in the plasma concentration of chloride and of potassium resulted necessarily in an increase in the amount of these ions filtered. After injection of the KCl solution the mean amount of chloride filtered per 100 g. body weight per min. was 1·39 mg., that of potassium 0·11 mg. and that of sodium 1·21 mg. (Table 1). The mean value for the rate of chloride reabsorption, expressed as percentage of the amount of chloride filtered was $T_{Cl} = 97{\cdot}4 \pm 0{\cdot}57\,\%$ (12), a figure which is significantly different from that obtained during a normal water diuresis ($t = 2{\cdot}258$, $P < 0{\cdot}05$). The mean value for the rate of sodium reabsorption was $T_{Na} = 98{\cdot}3 \pm 0{\cdot}28$ (12). The difference between the rate of chloride and of sodium reabsorption remained significant, $t = 2{\cdot}806$, $P < 0{\cdot}02$. It is thus clear that both chloride and sodium were reabsorbed at higher rates than during a normal water diuresis, but that, just as in control rats, the two rates were not the same. The rates of chloride and of sodium reabsorption,



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Such an injection resulted in an increase of potassium and of chloride in the plasma, and had a pronounced diuretic effect. The mean value for the G.F.R. was 0·41 ± 0·042 (12) c.c./100 g./min., a value which is comparable to that observed during a normal water diuresis. The mean plasma chloride amounted to 339·9 ± 6·42 (12) mg./100 g., and the plasma potassium was 27·2 ± 0·71 (12) mg./100 c.c., compared with 24·2 ± 0·28 in normal rats. However, the plasma sodium remained normal (293·5 ± 7·09 (12) mg./100 c.c.). The increase in the plasma concentration of chloride and of potassium resulted necessarily in an increase in the amount of these ions filtered. After injection of the KCl solution the mean amount of chloride filtered per 100 g. body weight per min. was 1·39 mg., that of potassium 0·11 mg. and that of sodium 1·21 mg. (Table 1). The mean value for the rate of chloride reabsorption, expressed as percentage of the amount of chloride filtered was $T_{Cl} = 97{\cdot}4 \pm 0{\cdot}57\,\%$ (12), a figure which is significantly different from that obtained during a normal water diuresis ($t = 2{\cdot}258$, $P < 0{\cdot}05$). The mean value for the rate of sodium reabsorption was $T_{Na} = 98{\cdot}3 \pm 0{\cdot}28$ (12). The difference between the rate of chloride and of sodium reabsorption remained significant, $t = 2{\cdot}806$, $P < 0{\cdot}02$. It is thus clear that both chloride and sodium were reabsorbed at higher rates than during a normal water diuresis, but that, just as in control rats, the two rates were not the same. The rates of chloride and of sodium reabsorption, expressed as percentages of the amounts filtered (T_{Cl} and T_{Na}) were unaffected by the rate of water reabsorption and that of urine flow, i.e. they remained practically constant at all rates of urine flow encountered.

In contrast to the rate of chloride and of sodium reabsorption, the rate of potassium reabsorption, expressed in percentage of the amount of potassium filtered (T_K) was found to be significantly correlated with the amount of potassium filtered $r = -0{\cdot}950$, $P < 0{\cdot}001$ (coefficient of regression, $b = 5{\cdot}27$) and with the rate of water reabsorption, $r = +0{\cdot}660$, $P < 0{\cdot}02$ (Table 1). A comparison with the data obtained during a normal water diuresis shows that the relationship between T_K and T_W was no longer the same ($r = +0{\cdot}660$ instead of $r = +0{\cdot}757$), but that the degree of relation between the amount of potassium filtered and the rate of potassium reabsorbed remained unaltered ($r = -0{\cdot}950$ and $r = -0{\cdot}940$).

Renal excretion of chloride, sodium and potassium after the administration of 85 mM *solution of* NaCl. Inulin, chloride, sodium and potassium clearances were estimated as usual. The weight of 85 mM solution of NaCl given by stomach tube was 5% of the rat's body weight. This caused a rise in the rate of water reabsorption and ultimately a decrease in the rate of urine flow (Dicker, 1946). The plasma chloride and sodium levels increased without affecting that of potassium.

The mean G.F.R. amounted to 0·94 ± 0·05 (11) c.c./100 g./min. The plasma chloride concentration was 322·6 ± 8·30 (11) mg./100 c.c., a figure which is comparable with that found in rats injected with KCl ($t = 1{\cdot}124$, $P > 0{\cdot}2$) and the plasma sodium level rose to 323·2 ± 7·13 (11) mg./100 c.c. The plasma potassium concentration was almost the same as that in rats during a water diuresis 23·4 mg./100 c.c. ($t = 0{\cdot}745$, $P > 0{\cdot}4$). The mean amount of chloride filtered per min. per 100 g. rat was 3·01 mg., that of sodium was 3·04 mg. and that of potassium 0·22 mg. (Table 1).

The rates of chloride and of sodium reabsorption expressed as percentages of the amount of chloride and of sodium filtered (T_{Cl} and T_{Na}) did not change with changes of urine flow and were independent of the rate of water reabsorption (T_W). The mean value for T_{Cl} was 97·8 + 0·32% (11) and that for T_{Na} 98·6 + 0·14% (11). These values do not differ significantly from those for T_{Cl} and T_{Na} in rats given KCl solution ($t = 0{\cdot}560$, $P > 0{\cdot}6$ and $t = 1{\cdot}869$, $P > 0{\cdot}05$, respectively) though the amounts of chloride and of sodium filtered were significantly higher (Table 1).

The rate of potassium reabsorption (T_K) was no longer significantly correlated with the rate of water reabsorption $r = +0{\cdot}365$, $P > 0{\cdot}1$. The amounts of potassium reabsorbed were much higher than in the other two series but remained closely correlated with the amount of potassium filtered $r = -0{\cdot}955$, $P < 0{\cdot}001$ (coefficient of regression, $b = -4{\cdot}89$) (Table 1).

DISCUSSION

The mechanism of renal excretion of potassium in rats has been found to be the same as that observed in dogs by Winkler & Smith (1942). During a normal water diuresis the amount of potassium reabsorbed proved to be closely correlated with the amount of potassium filtered. This correlation persisted when the amount of potassium filtered was raised either by increasing the level of potassium in the plasma or by increasing the glomerular filtration rate —as after administration of a 85 mM solution of NaCl. The mechanism of renal potassium excretion in rats stands thus in complete contrast to the mechanism of renal excretion of other ions like chloride and sodium.

SUMMARY

1. The renal excretion of chloride, sodium and potassium in rats was investigated with the aid of inulin clearances: (*a*) during a water diuresis, (*b*) after injection of 0·1 c.c./100 g. body weight of a 3·0% KCl solution, and (*c*) after administration of 5·0 c.c./100 g. body weight of a 85 mM solution of NaCl.

2. The rates of chloride and of sodium reabsorption were significantly different, and independent of the urine flow and of the rate of water reabsorption. No correlation could be found between the rate of chloride and of sodium reabsorption by the tubules and the amount of chloride and of sodium filtered.

3. During a water diuresis the rate of potassium reabsorption was correlated with the amount of potassium filtered per unit time. This correlation persisted when the amount of potassium filtered was raised either by increasing the level of potassium in the plasma or by increasing the glomerular filtration rate.

4. The reabsorption of potassium does not passively follow that of water.

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AN AUTOMATIC METHOD FOR THE COLLECTION OF THE ALVEOLAR AIR

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A method for the direct analysis of alveolar air was described by Haldane & Priestley (1905). In their procedure, the subject is instructed to breathe normally until the instant at which it is desired to take a sample of air, then to expire deeply. The last portion of expired air is collected and analysed. Modifications of their technique and apparatus have been published by Fridericia (1914) and by Henderson & Morriss (1917).

In practice, it is difficult to obtain valid alveolar samples, even with the co-operation of a trained subject, and with hospital patients it is almost impossible. Haldane & Priestley (1935) have stated that an expiration of the order of 800 c.c. is required adequately to wash out the respiratory dead space, and if this expiration be abnormally prolonged, the concentration of CO_2 in the alveoli will increase. There is, moreover, an instinctive tendency to preface a deep expiration with a deep inspiration, so that alveolar air is diluted with an abnormal amount of inspired air; furthermore, the fact of thinking about one's breathing tends to alter its depth and rate.

Henderson & Haggard (1925) sought to avoid the necessity for the voluntary co-operation of the subject. Their method was similar in principle to that of Krogh & Lindhard (1914), in which the sample is taken from the last part of a series of normal expirations, except that Henderson & Haggard made the sampling automatic by an ingenious valve system. However, it has been held against Krogh & Lindhard's method that a normal expiration is of insufficient depth to wash out the respiratory dead-space and that it does not therefore yield alveolar air. This criticism applies equally to Henderson & Haggard's technique and from figures quoted by Haldane & Priestley (1935) (Table 1) concerning the volume of air required to wash out the dead-space, it would seem that this is a fatal objection.

TABLE 1

Volume of air expired through tube (c.c.)	CO_2 in sample taken from tube (%)
190	3·03
335	4·37
510	5·04
650	5·19
950	5·51
1350	5·48

It should be noted, however, that even if this objection did not arise, the alveolar air obtained by the Henderson-Haggard method would not necessarily be strictly comparable with that obtained by the Haldane-Priestley technique. In the former case, what is obtained is a sample of which the composition is the mean over a given period of time of a series of end-of-expiration samples. It gives no information about the composition of the 'average alveolar air' which, strictly speaking, is the mean composition of the gases in the alveoli during a complete respiratory cycle or series of cycles. In the Haldane-Priestley method it is possible to take an alveolar sample at any desired phase of respiration.

It was felt that the criticisms levelled at Krogh & Lindhard's principle, i.e. that the last portion of an ordinary expiration yielded alveolar air, were cogent. It was therefore decided that an attempt should be made to make Haldane & Priestley's original technique automatic and independent of the co-operation or even of the prior knowledge of the subject. Accordingly, such an automatic apparatus (Text-figs. 1–3 and Pl. 1, figs. 1, 2) was evolved.

METHODS

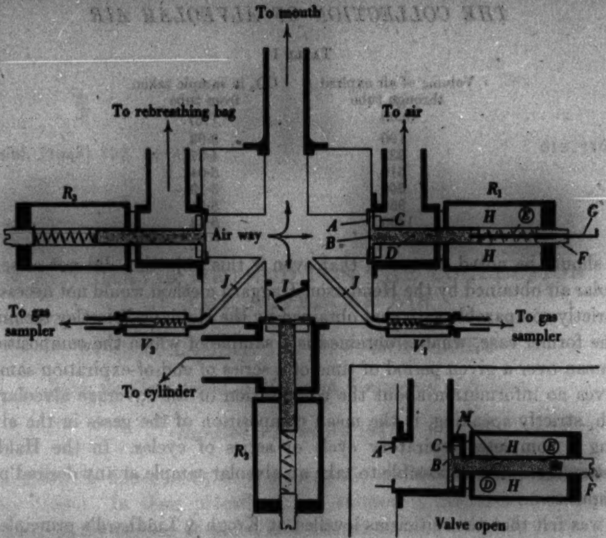
Description of apparatus. The central portion of this apparatus (Text-figs. 1 and Pl. 1, fig. 1) consists of an aluminium cube of side 2½ in. traversed by two airways ⅜ in. in diameter, at right angles to one another.

The whole block is mounted on a hospital-type bed-table and it is swung upon a pin so that it may pivot in a vertical plane. This is for convenience in adjusting the mouthpiece to the subject. The front of the block bears a metal tube carrying a rubber mouthpiece. Respiratory valves R_1, R_2, R_3 are attached to the block at those places where the other three limbs of the airway terminate. They are attached in such a fashion that the airway is continuous through the valve when it is open.

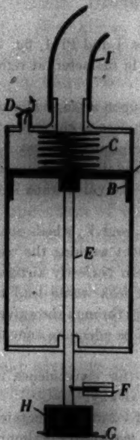
The essential components in the whole apparatus are the small valves V_1 and V_2 which open and shut the ports to the gas-samplers, for without their smooth and efficient working the rest of the apparatus would be pointless. It is essential that they should remain perfectly airtight against atmospheric pressure over quite a space of time, for the slightest leak would lead to contamination of the gas samples. For the same reason, the size of the airways through the valves and the connexions thereto have to be kept down to a minimum and yet to be adequate enough to permit of the almost instantaneous filling of the samplers.

The valves consist fundamentally of a coil solenoid surrounding, but (for convenience in renewal) readily detachable from a central duralumin tube which forms the airway.

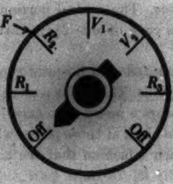
The coil has a length of approximately 1·25 in. and an overall diameter of 1·2 in. and contains about 600 turns of enamelled copper wire of 24-gauge. It fits snugly over the machined duralumin


To mouth
To rebreathing bag
To air
R2
R1
Air way
A
B
C
D
H
E
G
F
J
I
J
To gas sampler
To gas sampler
V2
V1
To cylinder
R3
M
Valve open

Text-fig. 1. Diagram of apparatus for the automatic switching of respired gases from one circuit to another and for the automatic sampling of alveolar air. Inset: valve open. R_1, R_2, R_3, respiratory valves; V_1, V_2, gas-sampler valves; *A*, rubber valve seating; *B*, base of iron plunger-type valve; *C*, rubber buffer; *D*, body of plunger valve; *E*, metal spring; *F*, metal stop for spring; *G*, extension for manually operating valve; *H*, coil solenoid; *I*, accessory valve; *J*, channel from gas-samplers to airway; *M*, main body of valve.



Text-fig. 2.


F
R2
V1
V2
R1
R3
Off
Off

Text-fig. 3.

Text-fig. 2. Device for drawing off tidal and supplemental air. *A*, cylinder; *B*, piston; *C*, spring; *D*, resetting valve; *E*, piston rod; *F*, solenoid release; *G*, tray for weights; *H*, weights; *I*, rubber hose connexion to R_3 (Text-fig. 1).

Text-fig. 3. Dial of rotary type switch showing connexions. When the pointer is turned to one of the letters on the dial the coil corresponding to it is instantly energized and the valve opens.

tube which has an internal diameter of 7 mm. in that section of it in which the plunger moves. That portion which screws into the aluminium block, and also the channel *J* leading to the main airway have an internal diameter of 2 mm. so as to reduce dead-space. The distal part of the duralumin tube which is connected with the gas-sampler has also an internal diameter of 2 mm. The proximal end of this portion of the tube is expanded into a flange 20 mm. in diameter and 2 mm. thick and this is kept in firm opposition with a similar flange on the distal end of the remainder of the duralumin tube by means of three screws.

The distal flange is machined to take a disk of oil-resistant synthetic rubber 10 mm. in diameter and having a centrally situated hole 1 mm. in diameter. This forms the valve seating and, when the coil is not energized, the soft-iron plunger is kept closely pressed against it by a spring. Both the plunger and the spring are chromium plated so as to prevent rusting. The plunger has a length of 22 mm. and a diameter such that it makes a smooth sliding fit in the duralumin tube and, in order to avoid any undue resistance to the air passing through the valve, it carries three shallow longitudinal channels. It is sparingly lubricated with light paraffin oil, as is also the rubber valve seating. Heavy pressure tubing, previously cleaned in acid and alkali and soaked in glycerine, provides attachment between the gas-sampler and the end of the valve. The dead-space herein can be obliterated either by filling with mercury through the side-arm of the sampler or by evacuating through the same way.

It was found that this type of valve maintained a vacuum indefinitely against atmospheric pressure, provided that the valve seating was kept clean and free from foreign matter. When the coil was energized, the soft-iron plunger, immediately becoming magnetized, lifted and slid along the tube compressing the spring and opening the valve. The already opened and evacuated gas-sampler filled in a fraction of a second. When the coil was de-energized, the plunger was again forced by the spring hard against the rubber seating, thus closing the valve. The gas-samplers themselves are supported on stands at an angle of about 45°, and still contained under Toricellian vacuum about 1 c.c. of mercury. This arrangement served two purposes: (*a*) as a check on the filling of the samplers when the valves are opened, and (*b*) as a check on the general overall efficiency of the valves, because the smallest bubble of air entering the gas-samplers disturbs the surface of the mercury and leads to the immediate detection of any leak.

The larger respiratory valves R_1, R_2, R_3 (Text-fig. 1) are constructed on the same general principle as the smaller valves, that is, a coil solenoid and a sliding soft-iron armature. They are much bigger, however, and in order to provide no resistance to the air breathed by the subject, they have an effective clearance in all directions of not less than ⅞ in. The function of these valves is to control the direction in which the respired air is passing. The coil solenoid *H*, as in the case of the smaller valves V_1 and V_2, is wound on a separate removable former and has a length of 2 in. and an overall diameter of 1¾ in. and contains some 1000 turns of 24-gauge enamelled copper wire. The main body of the valve *M* is machined from a casting in solid aluminium. Its base makes an air-tight joint with the central aluminium block, to which it is attached by three screws. The synthetic rubber valve seating *A* is held firmly in position between the base and the aluminium block.

The more distal part of the body of the valve *M* forms a tubular extension machined internally to a diameter of 0·6 in. The coil solenoid slips neatly over this and is held in place by a nut (not shown in diagram). Internally, the extension is machined to a diameter of 0·4 in. and forms a guideway for the soft-iron plunger *D* which makes a smooth sliding fit in it. The base *B* of the plunger takes the form of a disk 1 in. in diameter and when the valve is in the closed position it is pressed hard up against the seating *A* by the spiral spring *E*. The whole of the plunger is heavily plated to avoid rusting from the water vapour in the expired air. In order to enable the valve to be worked manually, a duralumin rod *G* is let into the distal end of the plunger *D*. Attached to *F*, the metal stop for the spring, though not shown in the diagram, there is a simple catch which holds the valve open mechanically. Should it be necessary to have the valve open for any considerable length of time, it is advisable to use this rather than to energize the coil, because prolonged energizing of the coil leads to a certain amount of 'heating up' which is transmitted by conduction to the rest of the apparatus.

The respiratory valve R_2 is connected by rubber hose 1 in. in diameter to the cylinder A in Text-fig. 2, the purpose of which is to draw off the tidal and supplemental air. The cylinder, which is of iron, is approximately 10 in. in length and 5 in. in diameter and internally is mirror-finished and provided with an aluminium piston B. The piston rod E passes through a bearing supported from the base of the cylinder. This bears a metal disk G about 3 in. in diameter attached to its lower end and serves to carry the weights H.

The upper face of the cylinder, besides the connexion to the respiratory valve R_2, also carries a small one-way valve D. This is to allow egress of air when the piston is adjusted at the beginning of an experiment. Although the inside of the cylinder is kept well lubricated with light oil there is still a certain amount of initial inertia which is overcome by the addition of the spiral spring C above the cylinder and the solenoid type release F. At the beginning of an experiment the piston is pushed up, thus compressing the spring until the catch engages with a notch in the piston rod. When the coil in F is energized, the release operates, and the spring gives the piston a small initial thrust sufficient to overcome its inertia.

One end of each coil is connected through the central aluminium block with the negative side of the power supply. This consists of a rectifying unit delivering 40 V. (d.c.) from the a.c. mains supply. The positive side is connected through a rotary switch (Text-fig. 3) with the other end of the coils. By turning the switch, the coils are energized in turn and the magnetic field set up causes the soft-iron armature to move, and the valve immediately opens. When the power is cut off, the action in closing the valve is just as positive and just as immediate.

The rotary type switch (Text-fig. 3) is connected by some 15 ft. of flexible wiring with the apparatus so that the latter is capable of remote control. If desired, the whole operation of taking a sample can be carried out with the patient in one room and the operator in another. The observation of the patient is carried out through a glass panel let into the door.

Method. The subject is placed in the semi-recumbent position on the bed and the table to which the apparatus (Pl. 1, figs. 1, 2) is attached is wheeled into position. Evacuated gas-samplers are attached to the smaller valves V_1 and V_2 (Text-fig. 1) by the rubber tubing provided. They are left closed. A brief description of the procedure to be carried out can now be very profitably given to the patient as it tends to offset 'stage-fright' and put him at his ease. In order that the rhythm, rate and depth of breathing shall be as normal and undisturbed as possible, the only instruction given to the subject is that he should think of anything but his breathing. His conscious co-operation is neither desired nor necessary. The valve R_1 to the atmosphere is opened and the mouthpiece placed in position. The nose clip is next applied. It is necessary, at this stage, to ensure that the patient is physically comfortable and quite relaxed and not straining either to reach the mouthpiece or to keep it in position.

The piston B (Text-fig. 2) is then pushed up so that the catch F engages. The weights H (1–2 lb.) are placed on the tray G. These are to ensure that the desired negative pressure is attained. The dead-space below the valve-seatings in V_1 and V_2 is now evacuated or filled with mercury or glycerol; in any case, this dead-space is very small having a volume of only 60 cu.mm. The gas-sampler taps connecting with the valves V_1 and V_2 are now opened. If the valve R_1 has been on manual control, it is now changed to electric control by turning the pointer of the rotary switch to the position on the dial of the rotary switch (Text-fig. 3) marked R_1. The operator takes up a position where he can observe the subject's respirations. This can be done either by direct observation or by observing the movement of the writer of a Krogh or Benedict metabolimeter attached through a two-way valve to the free limb of R_1. By this means, the precise phase of respiration at which it is desired to take the alveolar sample can be judged. After a waiting period of about 3–5 min., to permit the subject's respirations to settle down, the alveolar sample is taken at the desired stage of respiration. To do this, the pointer on the dial of the switch is turned from R_1 to R_2 and when the piston has fallen, immediately through V_1 and V_2; the time of this last operation being judged from the filling of the gas-samplers. When the switch is turned from R_1 to R_2 the coil on R_1 is immediately de-energized and the valve plunger D (Text-fig. 1) is thrust hard up against the seating A, thereby closing it. At the same instant, the coil on R_2 is energized and the plunger lifts, thus opening the valve. This valve communicates with the cylinder already

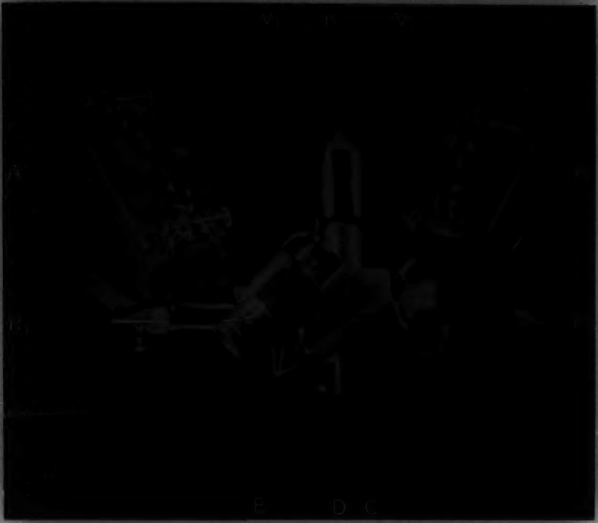


Fig. 1. Photograph of apparatus on hospital-type bed-table. R_1, R_2, R_3, respiratory valves; V_1, V_2, gas-sampler valves (V_1 obscured); *A*, gas-samplers on stands; *B*, valve attachment for taking Douglas bag specimens; *C*, aluminium block; *D*, mouthpiece.

Fig. 2. Apparatus with subject in position. The weights (*H* of Text-fig. 2) can be seen in lower left foreground. The cables in foreground lead to power unit and to the rotary switch for remote control.

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described (Text-fig. 2) and, at the same time as the coil on R_2 is energized, the coil on the piston release F (Text-fig. 2) is also energized.

The piston falls downwards under the influence of the weights and the slight initial impetus given to it by the spring above the piston. This causes air to be drawn out from the lungs into the cylinder, and the accessory valve I (Text-fig. 1), which is one-way acting, prevents any of this being drawn into the lungs again. The action of this combination though not instantaneous is very fast, occupying something less than a second. Its function is to draw out from the lungs as much of the tidal and supplemental air as will wash out the respiratory and instrumental dead-spaces and leave them filled with alveolar air.

When the switch is turned to V_1 and V_2 the coils attached here are energized and the valves are opened; but it is important to note that these valves do not operate simultaneously but consecutively: V_1 has opened and closed before V_2 is opened.

When the gas-samplers have been filled, the switch is turned to R_3, which is open to the air, and the patient is disconnected from the apparatus. The taps to the gas-chambers are turned off and they are removed to the Haldane gas-analysis apparatus for analysis of their contents. In the experiments recorded in Table 2 no special precautions were taken to obliterate the very small dead-space in the valves V_1 and V_2.

RESULTS

Some results obtained by the use of this apparatus are set out in Table 2. They are not selected in any way but are consecutive and the experiments were done as opportunity presented in the course of other work. The subjects for these experiments were students and members of the staff of this medical

TABLE 2. Alveolar tension (mm. Hg)

	CO_2		O_2		
	Gas-sampler (1st)	Gas-sampler (2nd)	Gas-sampler (1st)	Gas-sampler (2nd)	Remarks
W.A.G. ♂	39·0	38·9	100·2	96·0	Non-basal*
	40·4	40·2	100·7	100·5	„
H.N. ♂	40·2	40·2	94·8	95·0	„
B.J. ♂	40·4	40·2	109·6	108·6	„
	40·6	40·9	101·0	100·0	„
H.L. ♂	31·0	30·7	121·9	120·6	Non-basal after climbing stairs
	33·2	33·4	112·0	111·2	Non-basal
A.B. ♀	38·1	37·9	104·9	104·7	„
	37·4	37·4	109·8	108·9	„
	42·3	42·5	94·5	92·9	„
	38·7	38·7	104·0	104·0	„
	39·7	39·7	97·1	96·8	„
	43·0	43·0	93·4	92·7	„
	38·1	38·6	105·3	102·7	Non-basal†
W.T. ♂	43·1	43·1	101·0	100·8	Basal
	43·8	43·9	97·2	97·3	„
B.M. ♀	42·9	43·0	112·1	112·1	Non-basal
M.B. ♀	39·0	38·9	111·0	112·0	Basal
H.P. ♂	39·7	39·7	102·9	102·9	„

* An example of the case in which, though valid alveolar CO_2 samples were obtained, owing to the larger physiological dead-space for O_2 wide variation between oxygen tension in the two gas-samplers was obtained.

† Alveolar air obviously not obtained in this experiment.

school, but the apparatus has been used on hospital patients with similar results. For a reason presently to be discussed in detail, valid figures for alveolar CO_2 are more easily obtained than for O_2. Most of the figures are based on end of expiration samples but some were taken at 'end of inspiration' or even during inspiration.

DISCUSSION

By this system of valves, operated on the electromagnetic principle combined with the syringe-like action of the cylinder, it is possible to obtain alveolar samples from almost any patient.

The instrumental dead-space is low, amounting, as far as the airways in the aluminium block and mouthpiece are concerned, to less than 50 c.c. The dead-space of the gas-sampler valves is also very small indeed; that below the valve seating amounting to only 60 cu.mm. As already mentioned, this can be obliterated entirely. The volume of the dead-space above the valve seating is of the same order, but since it is open to the main airway at the moment of taking the sample, it is filled either with alveolar air or with respired air approaching it very closely in composition. The possibility of significant contamination is therefore small, particularly if the gas-samplers themselves have a fair capacity. The ones used in these experiments had a capacity of approximately 70 c.c.

These valves can be filled with glycerol if it is desired to obliterate the dead-space entirely, but it is doubtful whether this does lead, in actual practice, to greater accuracy. In routine experiments in this department, it was considered unnecessary to apply this extreme refinement in technique.

The action of all the valves, being practically instantaneous, is very much faster than a hand-operated valve could possibly be. The action is also much smoother and more positive—the valve is either fully open or fully closed.

The time taken in drawing off the tidal and supplemental air from the lungs and in taking the samples is determined by the speed at which the air is drawn into the cylinder and into the gas-samplers. The former can be judged from the rate at which the piston falls and can be varied by adding or removing weights from the tray on the end of the piston rod. The total time varies between 2 and 2·5 sec.

The question naturally arises as to whether alveolar air is obtained by this method. The criterion of validity used has been to compare the composition, as determined by the Haldane gas analysis apparatus, of the gas in the sampler attached to the valve V_1 with that in the sampler attached to the valve V_2. In the case of CO_2, should the composition in the first sampler be significantly less than that in the second, it was concluded that, as far as the first sampler was concerned, there was some contamination at least with non-alveolar air. As far as the second sampler was concerned, no valid conclusion could be drawn: it could or could not be alveolar air. Therefore all such results as these were rejected and the experiment was repeated. Should the second sampler contain significantly less CO_2 than the first (a phenomenon very rarely met with), it was concluded that some air had leaked inwards into the system with consequent contamination of the contents of sampler No. 2. No conclusion could be drawn about sampler No. 1 and, as before, the results were rejected and the experiment was repeated.

If, on analysis, the CO_2 content of each sampler was found to be the same (within the range of experimental error), it was concluded that they contained true alveolar air. If, however, the respiratory and instrumental dead-spaces had been incompletely washed out by the preliminary action of the cylinder, there would be an *increasing* CO_2 gradient backwards from the apparatus to the alveoli. The first sample drawn off would be immediately from the instrumental and buccal dead-spaces and would be replaced immediately with air from the deeper parts of the respiratory dead-space having a higher CO_2 content. The second sampler would be filled with this air and would have its higher CO_2 content.

In the case of O_2, however, since there is a decreasing O_2 gradient backwards from the apparatus to the alveoli, contamination from non-alveolar sources is indicated by a higher O_2 content in the first sample as compared with the second. In actual practice aberrant results were not often encountered.

It was sometimes observed that even when the CO_2 content of the first sampler agreed with that of the second—indicating that true alveolar air had been obtained—the O_2 content of the second sampler was lower than that of the first, indicating at first sight the contrary conclusion. However, this is only an apparent paradox, for it is possible to obtain a sample of 'alveolar air' having a correct CO_2 composition but an incorrect O_2 composition. The abovementioned observation is a confirmation of the fact pointed out by Haldane & Priestley (1905) that the physiological dead-space for CO_2 is smaller than it is for O_2 (Table 3).

TABLE 3

Mean depth of expirations at 37° C. (saturated) (c.c.)	Effective dead-space minus that of mouthpiece (c.c.)	
	Calculated from CO_2	Calculated from O_2
2984	683	920
2438	467	619
1413	272	392
650	171	223
643	161	231
410	111	136
357	185	199

(From Haldane & Priestley, 1935).

This being the case, it requires an expiration of much greater depth to wash out the O_2 dead-space than it does to wash out the CO_2 dead-space. If this greater depth is not attained, any 'alveolar air' collected will have the correct composition for CO_2 but, as far as O_2 is concerned, will have a composition somewhat higher than 'alveolar', since it is mixed with an unknown though small amount of inspired air. Samples taken consecutively will reveal this lack of homogeneity.

A point which is of some importance arises from the fact that the washing out of the respiratory and instrumental dead-spaces and the taking of the samples result in the production of a slight negative pressure within the lung-instrument system. This is never greater than 20 mm. Hg and is mostly much less. It can easily be observed by incorporating a manometer in the apparatus. The question arises, however, as to what figure should be used in computing the alveolar gas tensions: whether the prevailing barometric pressure or this figure less the observed negative pressure within the system. Hill & Greenwood (1905–6) showed that the product of the alveolar gas percentage and the prevailing pressure was, within experimental error, a constant, i.e. that the gas tension in the alveoli remained constant notwithstanding changes in pressure. However, in their experiments, ample time was allowed for a state of equilibrium to be reached. In the experiments herein described, the slight negative pressure is produced almost instantaneously. It is our opinion that the alveolar gas tension obtained should be related to the barometric pressure prevailing immediately before the samples were taken, that is, that the slight drop in pressure does not in any way reflect itself in the samples obtained. This opinion is based on the following points:

(1) Only a relatively small change in pressure is involved.

(2) The time during which it acts is short.

(3) The speed with which the piston withdraws the 'alveolar air' from the alveoli into the respiratory and instrumental dead-spaces is such that there is insufficient time for changes to take place in the composition of *this* alveolar air.

(4) The samples are drawn off from the 'alveolar air' already in the respiratory and instrumental dead-spaces and a change, even if it does occur, in the composition of the gas still in the alveoli cannot affect the composition of the gas already drawn off from the alveoli.

(5) If it were true that the 'alveolar air' as collected is influenced by the change in pressure it would *never* be possible to collect two consecutive samples having the same gaseous composition, for the drawing off of each sample further augments, though only momentarily, the negative pressure in the system. If the 'alveolar air' composition over this short space of time is proportional to the negative pressure, the sampler last filled would *always* contain more CO_2 than one taken off sooner, for the negative pressure increases with the tendency to diminution in volume of the lung-apparatus system. The fact is, however, that samplers filled consecutively according to this technique

do show, within experimental error, complete agreement in their gaseous composition and those deviations which are observed can be explained as due to other causes than change of pressure within the system.

SUMMARY

1. An apparatus is described for obtaining samples of 'alveolar air' without the co-operation or even the knowledge of the subject.

2. By taking consecutive samples and analysing them, it is possible to determine from the analytical results alone whether true 'alveolar air' has been obtained.

3. Attention is drawn to the fact first stated by Haldane & Priestley and supported by these experimental results that the respiratory dead-space is less for CO_2 than for O_2.

We wish to thank Dr A. J. Palmer for her assistance in the experimental part of the work. For the photographs and drawings, we are indebted respectively to Mr S. Woodward-Smith and to Mr R. B. Dunphy. Our thanks are also due to Mr L. P. Heffernan, without whose craftsmanship the apparatus herein described could not have been made.

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A NOTE ON THE SHAPE OF PIPETTES USED IN BLOOD GAS ANALYSIS

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During a series of blood gas analyses, attention was drawn to several points connected with the shape of the blood sampling pipettes. The well-known Ostwald blood pipette consists of a capillary tube about 2 mm. in diameter expanded about its mid-length into a bulb. It is calibrated *to deliver* the stated volume at the stated temperature between two etched markings, one above and one below the bulb. Frequently a tap is interposed between the lower mark and the tip.

After delivery of the blood sample from the Ostwald pipette, the length of capillary tubing between the lower mark and the tip is left filled with blood. At first sight it would appear that this provides protection against contamination with air because it seems likely that that blood which is left has originally been the blood uppermost in the pipette and therefore most liable to come in contact with the air. However, that this protection does not in fact exist is shown by the following experiments:

By suction at the tip, an inverted blood pipette was filled to the tap with distilled water. By further suction, a small volume of methylene blue solution was then drawn in. By this means, the movement of fluid in the capillary portion of the pipette was shown to be in the form of an axial spike (Fig. 1*a*). Henderson, Chillingworth & Whitney (1915) had noticed a similar phenomenon when air was passed along a narrow tube. The pipette was then turned right-way up, and the fluid run down until its surface was level with the upper calibration mark. It was observed that the axial current, as shown by the movement of the methylene blue solution, was still strongly evident down through the fluid in the bulb of the pipette (Fig. 1*b*). The axial stream still persisted when the fluid was run down to the lower calibration mark.

If the fluid was run down quickly through the pipette, its uppermost portion, as denoted by the methylene blue solution, passed straight through the pipette with little admixture with the distilled water, the bulk of which still remained in the bulb after all trace of the methylene blue had disappeared (Fig. 1*d*). The more slowly the fluid was run out, the more admixture occurred (Fig. 1*c*), but even at the slowest speeds the rapidity with which methylene blue reached the lower end of the pipette was surprising. Furthermore, the last portion of the fluid to drain from the bulb of the pipette was almost invariably distilled water uncontaminated with methylene blue.

From this, it is evident that the blood which remains in the pipette is not that which was originally the uppermost portion, for this blood is often among the first to be delivered by the pipette. The axial stream is just as fast and just as narrow across the bulb of the pipette as it is through the capillary portion, thus leaving a wide peripheral stream practically unmoving. Thus, the presence of the bulb accentuates this tendency for the uppermost portion of the fluid in the pipette to reach the lower end first.

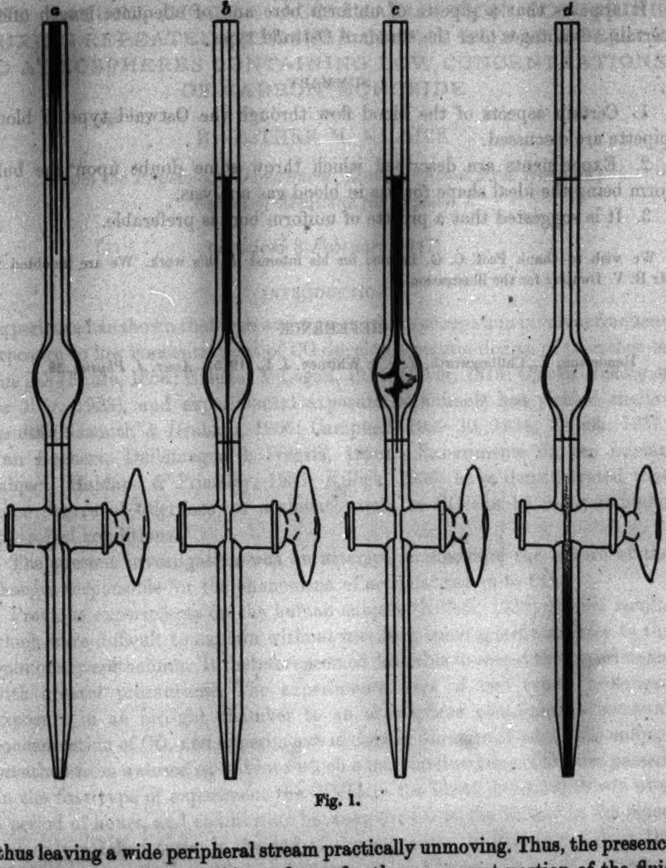

Fig. 1.

Also evident from these experiments is the fact that the motion of the axial stream tends to draw in continuously the layer forming the upper surface of the fluid. Thus, a new surface is all the time being exposed as the fluid descends in the pipette. This surface exposure is maximal in the bulb.

The purpose of the bulb is to make the pipette of manageable length, but its presence does, at least theoretically, increase the possibility of the contamination of the delivered sample of blood.

It appears that a pipette of uniform bore and of adequate length offers certain advantages over the standard Ostwald type.

SUMMARY

1. Certain aspects of the blood flow through the Ostwald type of blood pipette are discussed.
2. Experiments are described which throw some doubt upon the bulb form being the ideal shape for use in blood gas analysis.
3. It is suggested that a pipette of uniform bore is preferable.

We wish to thank Prof. C. G. Lambie for his interest in this work. We are indebted to Mr R. V. Dunphy for the illustration.

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THE NATURE OF THE ACCLIMATIZATION OCCURRING DURING REPEATED EXPOSURE OF THE HUMAN SUBJECT TO ATMOSPHERES CONTAINING LOW CONCENTRATIONS OF CARBON MONOXIDE

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INTRODUCTION

Experience has shown that men and women whose occupation involves frequent exposure to low concentrations of CO develop a certain degree of tolerance to this gas (Faure, 1856; Glaister & Logan, 1914; Oliver, 1916; *Official History of the War*, 1923), and experimental exposure of animals has yielded similar results (Nasmith & Graham, 1906; Campbell, 1929–30, 1934; Killick, 1937; Van Bogaert, Dallemagne & Wégria, 1928). Experiments on the human subject (Haldane & Priestley, 1935; Killick, 1936) have demonstrated that some degree of tolerance, or acclimatization, to CO can be induced under controlled conditions.

The present investigation was an attempt to elucidate the nature of the changes responsible for the phenomena of acclimatization to CO.

Previous experiments on the human subject (Killick, 1936) yielded results which were difficult to explain without ascribing some specific activity to the pulmonary epithelium. It therefore seemed desirable to repeat the experiments with greater precautions. The experiments were of two types, prolonged exposure in an airtight chamber to an atmosphere containing a constant concentration of CO, and experiments of shorter duration in which the subject breathed from a closed circuit into which a measured volume of CO was passed. In the first type of experiment the COHb in the blood increased slowly over a period of hours, and equilibrium between the CO in the air and in the blood was only reached towards the end of the exposure. In the second type, the COHb rose rapidly; equilibrium between air and blood was quickly attained and the proportion of COHb then remained constant for the remaining period of the experiment (40–60 min.). Two important changes were made in the general plan of the experiments; all the blood samples were examined by another person as well as by the experimental subject in order to check the accuracy of the determination of CO in the blood. The second change concerned the determination of alveolar oxygen; in this more recent series of experiments alveolar air samples were taken towards the end of each experiment, so that accurate figures for the partial pressure of O_2 in the alveolar air were available for each experiment. Exposure to a constant concentration of CO was repeated at regular intervals of 5–8 days in order to produce some degree of acclimatization. The shorter closed-circuit experiments were planned as a test of the degree of acclimatization. Since equilibrium was rapidly attained between the CO in the circuit air and in the blood, the partial pressure of CO in the circuit air as determined by analysis could be related to the proportion of COHb in the subject's blood.

The relationship is expressed by the equation

$$\frac{\text{COHb}\,\%}{\text{O}_2\text{Hb}\,\%}=K\,\frac{\text{p.p. CO}}{\text{p.p. O}_2},$$

where p.p. O_2 is the partial pressure of O_2 in the alveolar air. The development of acclimatization has been shown (Killick, 1936) to alter this relationship, so that the observed proportion of COHb in the subject's blood becomes lower than the proportion calculated from the above equation, when the partial pressures of CO and O_2 are known. Thus the closed-circuit experiments should serve as a test of the degree of the subject's acclimatization. They also provide the data necessary for the calculation of blood volume.

METHODS

In general, the method of exposing the human subject to an atmosphere containing a constant concentration of CO was the same as that employed in the earlier series (Killick, 1936). The subject entered an airtight chamber of 10,000 l. capacity at about 10.30 a.m., and, as soon as the door was secured, a measured volume of pure CO was released into the air by water displacement. A small electric fan ensured thorough mixing of the air. The subject remained seated throughout the 6–7 hr. period of each experiment, except for the movements required to take blood and air samples and to use the reversion spectroscope. Blood samples were taken from a finger prick at hourly intervals, and the CO content of each was estimated in duplicate by an observer outside the chamber and by the experimental subject inside, both using the reversion spectroscope (Hartridge, 1912). The subject recorded her pulse rate, and any symptoms experienced during the period of exposure. Air samples were taken about 30 min. after the CO had been released, and about 5 hr. later; the CO in these samples was determined by the iodine pentoxide method in the apparatus devised by Graham (Haldane & Graham, 1935).

During the last ½ hr. of some of the exposures, a sample of air was taken for the determination of CO_2 and O_2 in the Haldane gas analysis apparatus. Samples of alveolar air were also taken about the same time; these samples were taken in evacuated tubes, using the Haldane-Priestley tube.

The method of collecting alveolar air was modified during this series of experiments. During the first fifteen experiments single samples were taken into evacuated sampling tubes of 25 c.c. capacity, one sample being taken after a forced expiration following normal inspiration, and the other after a forced expiration following normal expiration. The average O_2 % of these two samples seemed to be rather low, and therefore a modification of technique was adopted. Sampling tubes of 50 c.c. capacity were filled with Hg and attached to the Haldane-Priestley tube; one sampling tube was used for inspiratory samples and the other for expiratory samples, but four individual samples were taken into each tube by lowering the Hg to a suitable level (Campbell, Douglas & Hobson, 1914). This series of experiments comprised twenty-three exposures.

Blood examinations. On the day following each exposure to CO, red cell and reticulocyte counts were made on the subject's capillary blood.

Three determinations were made of the affinity constant of the subject's blood for CO, i.e. the value of K in the equation expressing the partition of haemoglobin between CO and O_2.

$$\frac{\text{COHb}\,\%}{\text{O}_2\text{Hb}\,\%} = K\,\frac{\text{p.p. CO}}{\text{p.p. O}_2}. \qquad (1)$$

Venous blood was drawn and at once defibrinated by whipping. Portions (0·2 c.c.) of this defibrinated blood were then placed in a series of 500 c.c. tonometers. Special precautions were taken to prevent drying of the blood during the subsequent period of equilibration. Expired air was breathed into the dry tonometer so that it contained water vapour to approximate saturation at body temperature. Some of this vapour immediately condensed on the walls of the tonometer, but of course vapourized again when the tonometer was brought to 37° C. for equilibration. The expired air was then rapidly sucked out, the measured volume of blood put in and allowed to spread in a ring round the tonometer wall, and the tonometer stoppered. CO was then added in volume sufficient to give the desired partial pressure, the volume being accurately measured by delivering the CO from a finely graduated special burette in which it was confined over Hg.

The tonometers were then rotated at 37° C. for 60 min., an air sample was taken and analysed for O_2 and CO to check the partial pressures, and the blood was diluted in the tonometer by passing in 10 c.c. ammoniacal distilled water without admitting air. The resulting haemoglobin solution was examined with the reversion spectroscope and the proportion of COHb determined.

The volume of the circulating blood was determined by the dye method (Keith, Rowntree & Geraghty, 1915) on three occasions, twice before the exposures to CO began, and once after acclimatization had developed.

Inhalation of measured volumes of CO. These experiments were carried out at intervals before and during the period occupied by the experiments in the airtight chamber, and were continued during the ensuing year. The subject breathed from a closed circuit (Douglas & Haldane, 1912) of total capacity about 15 l.; the CO_2 was absorbed by caustic coke, and O_2 was supplied through a Müller valve to maintain a constant volume.

A measured volume of pure CO was slowly passed into the circuit (at the rate of 15 c.c./min.) and the experiment was continued for about 80 min. after all the CO was in the circuit. Blood samples for the estimation of COHb were taken after 30 and 70 min. Air samples were taken for estimation of CO_2 and O_2 at 30, 50 and 70 min. Two large air samples for the estimation of CO were taken slowly by water displacement between the first and second small air samples. Just before the end of the experiment two samples of alveolar air were taken; inspiratory and expiratory. The samples were taken through a side tube just beyond the expiratory valve, and were timed in the same way as is employed in the Haldane-Priestley method, the sample being taken during the inspiration following the deep expiration. At the end of the experiment another air sample was taken for the estimation of CO. The following protocol shows the arrangement of a typical experiment:

Closed circuit experiment—15. 6. 39

Hb estimation for O_2 capacity

$$\left.\begin{array}{l}\text{Haemoglobinometer } x\ 78\,\% \equiv 16{\cdot}13 \text{ vol. }\% \\ \text{Haemoglobinometer } c\ 81\,\% \equiv 16{\cdot}42 \text{ vol. }\%\end{array}\right\}\ 16{\cdot}28 \text{ vol. }\%\ \text{O}_2.$$

10.27 a.m.	Started breathing from circuit	
10.30 a.m.	CO started	
10.41 a.m.	170 c.c. CO into circuit	
11.11 a.m.	1st small air sample from circuit	19·3 % O_2

11.13 a.m.	1st blood sample	26% COHb
11.15 a.m.	1st large air sample from circuit	0·0195% CO
11.34 a.m.	2nd small air sample from circuit	18·3% O_2
11.36 a.m.	2nd large air sample from circuit	0·0195% CO
11.54 a.m.	3rd small air sample from circuit	20·4% O_2
11.55 a.m.	2nd blood sample	26% COHb
11.58 a.m.	Alveolar air sample—inspiratory	10·0% O_2
11.59 a.m.	Alveolar air sample—expiratory	11·6% O_2
12.00 noon	End of experiment	0·0195% CO

Barometer, 743·4 mm.; temperature, 18° C.

Before each experiment the subject's haemoglobin was estimated by the Gower-Haldane method. Data were therefore complete for the calculation of the circulating blood volume on the basis of the saturation of the blood with CO. The gas and blood analyses were carried out by the methods employed in the chamber experiments, O_2 and CO_2 being estimated in the Haldane gas analysis apparatus. Estimations of COHb were performed in duplicate by two observers and neither observer knew what his readings were until the determination was completed.

RESULTS

Exposure to constant concentration of CO *in an airtight chamber*

The results of this series of experiments confirmed those of the previous series (Killick, 1936). In general, the estimation of COHb performed by the observer outside the chamber yielded results very close to those obtained by the experimental subject (see Fig. 3); in the experiments where a discrepancy appeared between the two, greater reliance was placed on the results obtained by the observer outside.

The concentration of CO in the air of the chamber remained constant throughout each experiment, the variation between the samples taken near the beginning and near the end of the experiment being usually within the limits of experimental error. At the end of an experiment the O_2 in the air had usually diminished to about 20%, while the CO_2 had increased to about 1%. This alteration in the inspired air did not appear to affect the breathing, the rate and depth of which is not usually appreciably increased until the CO_2 in the inspired air reaches about 2% (Davies, Brow & Binger, 1925). The small decrease in O_2 might be the explanation of the somewhat low values recorded for alveolar O_2 (see Table 1). As already described, these low values during the earlier experiments of the series threw some doubt on the accuracy of the method and led to its modification. Reference to Table 1 will show that the composite samples of alveolar air indicated a higher O_2 content than the single samples.

In an attempt to explain this discrepancy in the results obtained by the two methods a series of alveolar air analyses was done when the subject was in the normal resting state without any CO. This series also showed higher values for alveolar O_2 determined in composite samples than in paired single samples, the difference being from 0·10 to 0·95% O_2; when the same number of individual samples was taken, however, that is four separate inspiratory and four separate expiratory samples, the average of the four pairs of results agreed very closely with the result of analysing the composite samples.

TABLE 1. Exposure to constant concentration of CO

Date	% CO in air	Duration of experiment		COHb % at equilibrium		p.p. O_2		p.p. CO	
		Hr.	Min.	Obs.	Calc.	Alveolar air (mm. Hg)	Arterial‡ blood (mm. Hg)	Alveolar air (mm. Hg)	Arterial§ blood (mm. Hg)
2. xii. 37	0·019	6	—	26·5	26	89·0	86·5	0·13	0·13
7. xii. 37	0·020	6	20	27	28	88·5	90·5	0·14	0·14
15. xii. 37	0·027	6	15	30·5	34	87·5	104	0·19	0·16
22. xii. 37	0·029	6	15	29	35·5	90·0	121	0·21	0·15
4. i. 38	0·028	6	20	30	35	89·0	113	0·20	0·16
13. i. 38	0·029	6	20	29·5	36	87·7	117	0·20	0·15
18. i. 38	0·035	6	10	33	41	86·1	122	0·25	0·18
26. i. 38	0·035	6	20	37	39·5	91·0	101	0·25	0·22
2. ii. 38	0·035	5	30	35	40·5	89·6	112	0·25	0·20
8. ii. 38	0·034	6	15	32	40·5	86·0	125	0·24	0·17
17. ii. 38	0·036	6	10	39	41	89·0	97	0·26	0·24
22. ii. 38	0·036	6	15	37·5	40·5	90·6	104	0·26	0·23
1. iii. 38	0·040	6	10	—*	42·5	90·3	—	0·29	—
8. iii. 38	0·040	6	15	39·5	43·5	87·5	103	0·28	0·24
14. iii. 38	0·041	6	15	36	44	88·4	124	0·29	0·21
22. iii. 38	0·045	6	—	38	45	92·8†	124	0·32	0·24
29. iii. 38	0·022	6	20	16·5	29	92·8	191	0·16	0·08
5. iv. 38	0·032	6	20	27	37	93·7	148	0·23	0·14
3. v. 38	0·032	6	10	27·5	37·5	89·7	142	0·23	0·14
10. v. 38	0·022	6	10	19	28	98·6	163	0·16	0·10
17. v. 38	0·040	6	20	33	40·5	97·1	134	0·28	0·20
24. v. 38	0·023	6	20	19	28	96·4	162	0·16	0·09
31. v. 38	0·030	6	15	27	34·5	95·5	135	0·21	0·15

* COHb still rising at the end of exposure.
† Alveolar O_2 determined on composite samples from this date onwards.
‡ Assuming that p.p. CO is the same in alveolar air and arterial blood at equilibrium.
§ Assuming that p.p. O_2 is the same in alveolar air and arterial blood.

The method used to assess the degree of acclimatization was to calculate the proportion of COHb which the blood would contain if it were brought into equilibrium in vitro with the alveolar air, and to compare this value with the observed proportion of COHb in the subject's blood at the end of an experiment, when the COHb had reached a steady value. In making these calculations equation (1) expressing the partition of haemoglobin between O_2 and CO was used. Several assumptions were made. First, that no reduced Hb was present in the arterial blood in the conditions of the experiment, and therefore $O_2Hb = 100 - COHb$. Secondly, that the proportion of COHb was the same in the arterial blood as in the capillary blood; this assumption is justified if no CO is utilized by the tissues, and when the condition of equilibrium is considered. Thirdly, that the percentage of CO was the same in the alveolar air as in the inspired air; this, too, is justified when equilibrium has been attained. The value of *K* for the subject's blood was ascertained experimentally, as

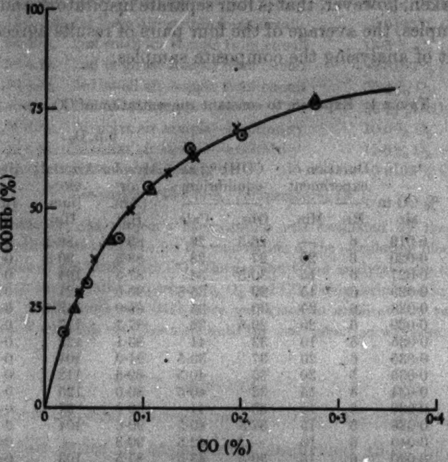

Fig. 1. Dissociation curve of COHb in presence of air (20·9 % O_2) at 37° C. ⊙ points determined before acclimatization 17 November 1936; × points determined before acclimatization 29 November 1937; △ points determined during acclimatization 24 June 1938.

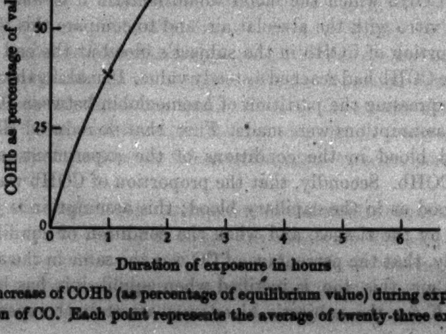

Fig. 2. Rate of increase of COHb (as percentage of equilibrium value) during exposure to constant concentration of CO. Each point represents the average of twenty-three experiments.

already described. It was found to be unchanged since the earlier series of experiments (Killick, 1936) and Fig. 1 shows that three determinations of the value of this constant made during the present series of experiments can be represented by a single curve, giving the value of K as 240.

During each experiment the proportion of COHb in the capillary blood was plotted against the time, and the resulting curves were all of a similar type as shown in Fig. 2, in which the COHb has been expressed as a percentage of the amount present in the capillary blood at the end of the exposure. Since each experiment was continued until it appeared that the COHb in the blood had reached a steady level, Fig. 2 may be assumed to show the percentage of the final equilibrium value that was attained after any given period of exposure.

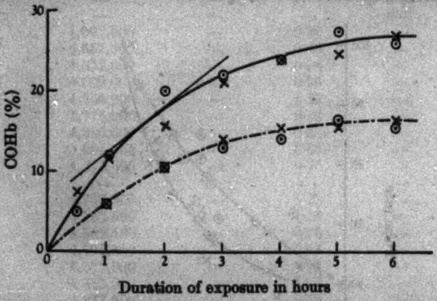

Fig. 3. Rate of increase of COHb during exposure to constant concentration of CO. — 2nd exposure (7 December 1937) to 0·020 % CO; –·–·– 17th exposure (29 March 1938) to 0·022 % CO, showing acclimatization. ⊙ subject's observations; × assistant's observations (outside airtight chamber).

The rate of absorption of CO seems to be somewhat slower in these experiments than in the earlier ones (Killick, 1936); these later results are probably more accurate, since they represent the average data of two sets of observations, and also because greater care was taken by the subject not to inhale the CO as it was liberated and before it was thoroughly mixed with the air of the chamber. The suggestion that the slower rate of absorption is nearer the truth is supported by a rough calculation of the volume of CO that would be absorbed during the first hour of exposure on the assumption that half the CO inhaled is absorbed (Haldane, 1895; Veale, 1922). During the first experiment, the Hb was just under 9 % saturated at the end of 1 hr. To obtain this degree of saturation while breathing 0·019 % CO the pulmonary ventilation would have to be about 8 l./min., which is quite a reasonable figure for slight activity.

At the beginning of the series of exposures the proportion of COHb in the blood when equilibrium was reached agreed closely with the value calculated (see Table 1). As the series progressed the proportion of COHb at equilibrium fell more and more markedly below this calculated value (see Table 1), the discrepancy being much the same whether the air concentration of CO was high or low. The curves relating COHb to time of exposure during each experiment were all of the same form, and the points from which the curve in Fig. 2 was constructed represent average values for the whole series of exposures. The degree of acclimatization attained is shown graphically in Fig. 3, in which the 2nd and 17th exposure are compared, the concentrations of CO in the air being 0·020 and 0·022%.

In this series of exposures an attempt was made to induce acclimatization without using concentrations of CO sufficiently high to cause unpleasant symptoms, but this was not very successful; definite acclimatization was attained only after exposure to 0·04% CO.

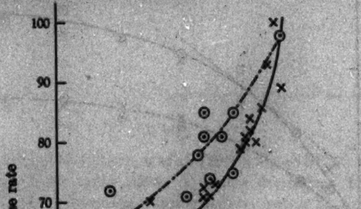

Fig. 4. Pulse rate during exposure to constant concentration of CO. ⊙—·—⊙ experiments showing little or no acclimatization; ×—× experiments showing definite acclimatization.

Pulse rate. Before counting the pulse the subject sat quiet for at least 5 min. to obtain the resting value. This was normally about 60 beats/min. The rate increased as the proportion of COHb in the blood increased, but the change was not large until the blood was 15–20% saturated with CO. At 20% saturation the pulse rate was about 70 beats/min., at 30% saturation it was 80 beats/min., while the rate rose more steeply at higher blood saturations, reaching 95–100 beats/min. at about 38% saturation.

If the pulse rate is plotted against the proportion of COHb in the blood, ignoring the air concentration of CO, there is some indication that the range of pulse rate was lower in the partially acclimatized than in the non-acclimatized subject, though the difference is probably not significant (Fig. 4).

Blood changes. Red cell counts, performed on the day after exposure to CO, showed fluctuations during the period of 6 months, varying from 4 to 5 million cells/cu.mm., but there was no regular increase or decrease in the number of cells, nor was any connexion apparent between the value of the count, and the symptoms shown during the previous day's experiment.

The proportion of reticulocytes also varied somewhat, but all the figures are within the limits usually accepted as normal, 0–2% of the red cells (Table 2).

TABLE 2. Blood examinations during series of exposures to constant concentrations of CO

Date	Red cells/cu.mm.	Hb %*	Reticulocytes %	Blood volume Dye (l.)	Blood volume CO (l.)
30. x. 36	—	—	—	3·82	—
13. xi. 37	—	—	—	4·65	—
1. xii. 37	4,067,500	—	0·30	—	—
3. xii. 37	4,657,500	—	0·80	—	—
9. xii. 37	4,572,000	—	0·94	—	—
16. xii. 37	4,502,000	—	0·96	—	—
23. xii. 37	4,760,000	—	1·23	—	—
5. i. 38	4,775,000	—	0·93	—	—
14. i. 38	4,395,000	—	0·58	—	—
19. i. 38	5,004,500	—	0·83	—	—
27. i. 38	4,572,500	—	0·73	—	—
31. i. 38	—	83	—	—	3·90
3. ii. 38	4,937,500	—	1·23	—	—
9. ii. 38	4,762,500	—	1·28	—	—
18. ii. 38	4,562,500	—	0·98	—	—
23. ii. 38	4,792,500	—	0·87	—	—
2. iii. 38	5,015,000	—	1·01	—	—
9. iii. 38	4,575,000	—	1·07	—	—
15. iii. 38	4,837,500	—	0·84	—	—
16. iii. 38	—	75	—	3·97	—
23. iii. 38	4,570,000	—	0·96	—	—
25. iii. 38	—	—	—	4·48	—
30. iii. 38	5,047,500	—	1·06	—	—
6. iv. 38	4,587,500	—	0·92	—	—
11. iv. 38	—	79	—	—	3·56
4. v. 38	4,345,000	—	1·14	—	—
11. v. 38	4,525,000	—	0·87	—	—
18. v. 38	4,522,500	—	0·86	—	—
25. v. 38	4,447,000	—	0·64	—	—
1. vi. 38	4,362,000	—	0·77	—	—
10. vi. 38	—	76·5	—	—	3·80
16. vi. 38	—	73	—	—	3·94
30. vi. 38	—	73	—	—	3·91

* 100% Hb is equivalent to 20 vol. % O_2.

Hb estimations were not made regularly with the red-cell counts, but they were made on the occasion of each closed-circuit experiment. It is of interest that the six estimations during the period of the later chamber experiments average 76·5% Hb, while sixteen estimates before the chamber experiments were begun averaged 87% Hb; and after the last chamber experiment there is some indication of a gradual rise in Hb content, five estimations made approximately a year later average 80·5% Hb. The differences are small, and may be quite independent of the exposures to CO.

The two determinations of blood volume by the Congo-red method (Keith, Rowntree & Gerachty, 1915), made before the exposures to CO were begun, gave an average of 4·24 l.; a third determination, after acclimatization had developed, gave a value of 4·48 l. Six determinations by the CO method (closed-circuit experiments) during the period of the later chamber experiments yielded an average of 3·85 l. (variation 3·56–3·97 l.). This average of 3·85 l. represents 69 c.c./kg. body weight. A series of sixteen similar experiments performed about a year before the chamber experiments gave an average value for blood volume of 3·87 l. (variation 3·20–4·62 l.).

Examination of the blood thus revealed no changes suggesting either increased cell destruction or formation during repeated exposures to low concentrations of CO. There was no evidence that acclimatization to CO was accompanied by an alteration in blood volume.

Symptoms. The symptoms experienced by the subject were similar to those described during an earlier series of experiments (Killick, 1936) and corresponded with the proportion of COHb observed in the blood; e.g. the first experiment was with a concentration of 0·019 % CO and the blood became 26·5 % saturated with CO; the 19th experiment was with a concentration of 0·032 % CO, and produced a blood saturation of 27·5 %. The symptoms during these two experiments were nearly identical; drowsiness and slight headache were noted after 3½–4 hr. exposure, with moderate headache and slight nausea during the recovery period; the pulse rate during each experiment increased by about 10 beats/min. If, on the other hand, two exposures to the same air concentration of CO are compared, the results are very different. The second experiment was with a concentration of 0·020 % CO, and the final blood saturation with CO was 27 %; the 17th experiment was with a concentration of 0·022 % CO, but the blood saturation reached only 16·5 %. When the blood was 27 % saturated in the earlier experiment the symptoms were similar to those described above, and, during recovery, bodily movement induced severe headache; in the later experiment, when the blood was 16·5 % saturated, there were no symptoms during exposure, and, after exposure, brisk exercise caused only slight transient headache.

Inhalation of measured volume of CO

A total of seventeen experiments is included in this series, extending over a period of 2 years and 5 months. Some experiments have been excluded because the O_2 in the circuit varied too much, and these experiments will be discussed later. In twelve of the seventeen experiments under discussion the O_2% in the circuit varied by less than 1·3, i.e. it was constant ± 0·65; in the other five the variation was 3·4, 2·4, 2·4, 2·1, 2·0. The results of these experiments are shown in Table 3. Breathing from a closed circuit of 15 l. capacity allows rapid attainment of equilibrium between the CO in the air of the circuit and the CO combined with Hb in the blood. This rapid attainment of equilibrium was demonstrated by the close agreement between air and blood samples taken respectively after 30 and 70 min. rebreathing. The position of this equilibrium, while the subject was acclimatized, did not correspond with that of the equilibrium attained in vitro with the same gas mixture. As in the experiments in the airtight chamber, the concentration of CO in the air was higher than would be expected from the observed degree of saturation of the Hb with CO. The discrepancy was similar in degree in the two types of experiment.

TABLE 3. Inhalation of CO from closed circuit

	Circuit air						Alveolar air		Calculated values		
	% CO after		% O_2 after		% COHb after		p.p. CO (mm. Hg)	p.p. O_2 (mm. Hg)	COHb* %	Arterial CO, p.p. in mm. Hg†	Arterial O_2, p.p. in mm. Hg‡
Date	30 min.	70 min.	30 min.	70 min.	30 min.	80 min.					
26. i. 37	—	0·016	19·8	19·6	27	27·5	0·11	77	26	0·12	70
3. iii. 37	—	0·018	19·9	17·9	32·5	33	0·13	64	32	0·13	63
4. v. 37	—	0·018	19·9	19·4	29·5	29	0·13	78	28·5	0·13	76
11. v. 37	—	0·026	21·2	20·3	35·5	35	0·17	75	35·5	0·17	76
31. i. 38	—	0·016	17·9	18·6	20	20	0·11	72	27	0·07	106
11. iv. 38	—	0·035	24·1	24·1	27·5	28	0·25	110	35	0·18	155
10. vi. 38	—	0·032	23·6	23·0	26·5	25·5	0·23	102	35	0·15	154
16. vi. 38	—	0·030	19·8	20·3	24	24·5	0·21	98	34	0·13	157
30. vi. 38	—	0·027	21·5	20·5	24·5	24·5	0·19	88	34	0·12	137
27. x. 38	—	0·019	22·6	19·2	28·5	28·5	0·13	76	29	0·13	79
4. xi. 38	0·015	0·015	16·9	14·5	22	23	0·10	54	31	0·07	87
12. i. 39	0·016	0·014	15·1	15·1	27	25	0·09	44	33	0·07	64
20. iv. 39	0·018	0·018	20·1	21·2	21	21·5	0·13	98	24	0·11	115
3. v. 39	0·018	0·018	19·3	19·1	24·5	24·5	0·13	86	26·5	0·12	95
4. v. 39	0·016	0·016	18·0	17·4	22	21	0·11	65	29	0·07	95
15. vi. 39	0·020	0·020	19·3	20·4	26	26	0·14	83	29	0·12	93
29. vi. 39	0·017	0·017	22·4	21·2	20	21·5	0·12	95	23	0·10	106

* Assuming that p.p. of O_2 and CO at equilibrium are the same in alveolar air and arterial blood.

† Assuming that p.p. of O_2 is the same in alveolar air and arterial blood.

‡ Assuming that p.p. of CO is the same in alveolar air and arterial blood at equilibrium.

The results obtained in this series of experiments with a closed respiratory circuit do not confirm those previously published (Killick, 1936). The methods employed in the present series were improved in several important respects and the results are, therefore, regarded as more reliable. In the earlier series the concentration of CO in the air of the circuit was estimated by combustion in a Haldane gas analysis apparatus. This method is not very satisfactory for low concentrations of CO, and was replaced by analysis in Graham's iodine pentoxide apparatus during the present series.

In the earlier experiments no regular estimations were made of O_2 in the air of the circuit, since this information was not directly needed for the purpose of the experiments. The present series of experiments was designed to test the validity of various explanations of the phenomena of acclimatization and it was necessary to know the O_2 content of the inspired air, and also to ensure that it varied only within narrow limits. The attempt to maintain the O_2

constant revealed how easily a very slight leak can cause a lowering of the O_2 content. The circuit was tested by fixing the mouthpiece tube of the valve box under mercury, attaching a water manometer to one of the side tubes, and leaving it overnight and with a slight negative pressure in the circuit. It was not used for experiment on the following day unless the negative pressure had been maintained. Regular estimations of O_2 during an experiment yield the best evidence that the circuit is really airtight, and since these estimations were lacking in the earlier experiments we cannot be certain that the circuit was always completely airtight.

Regarded as a test of the degree of the subject's acclimatization, the results of these experiments are interesting. The first four closed-circuit experiments were performed before the series of exposures in the airtight chamber began. These four experiments show no acclimatization, the observed proportion of COHb in the subject's blood being the same as that calculated from the observed alveolar oxygen and the concentration of CO in the circuit air. The next experiment, between the 8th and 9th exposures in the airtight chamber, showed some degree of acclimatization. The next was between the 18th and 19th exposures in the chamber, and showed the greatest degree of acclimatization; the remaining closed-circuit experiments were done after the chamber exposures had ceased, and they show a decreasing degree of acclimatization. It is interesting that on several occasions when two closed-circuit experiments were performed within a few days of one another the degree of acclimatization was greater on the second occasion. Acclimatization was not completely lost when the last closed-circuit experiment was made 13 months after the final chamber experiment.

Some interesting results were observed in experiments where the O_2% in the closed circuit diminished progressively instead of remaining constant. In Fig. 5 the O_2% in the inspired air is plotted against that in the alveolar air, and it will be seen that the relationship agrees closely with that observed by Dill and his co-workers (Bock, Dill, Edwards, Henderson and Talbott, 1929). In a few experiments which were terminated before samples of alveolar air had been obtained, an approximate value for alveolar O_2 may be read from this curve.

When the alveolar O_2 fell below 6·5% (46 mm. Hg) slight symptoms of anoxaemia began to appear when the subject was at rest; below 5% (35 mm. Hg) alveolar O_2 these symptoms became more marked, and on three occasions when the subject lost consciousness the O_2 in the inspired air was about 9% and in the alveolar air probably about 4% (28 mm. Hg). During some earlier experiments the subject did muscular work, and under these conditions of moderate exercise there was tremor and dizziness when the inspired air contained 10·7% O_2 and the alveolar air 5·0% O_2 (35 mm. Hg). These results are of interest in relation to the question of the partial pressure of O_2 in the arterial blood when the subject was acclimatized to CO.

The symptoms associated with a low O_2 content of the inspired air seemed to be due to the lowered partial pressure of O_2 in the alveolar air and in the arterial blood, and not to the presence of COHb in the blood, for during relatively short (up to 2 hr.) closed-circuit experiments a blood saturation of 25–30% COHb did not appear to cause any definite symptoms when the O_2 content of the inspired air was normal. Two experiments made with no CO, to test the circuit, confirm this; in one the O_2 in the circuit fell to 11·5% without causing any symptoms, in the other it fell to 8·9% and the subject experienced marked tremor (evidenced in the handwriting) and such dizziness that the experiment was ended. Apparently symptoms of anoxaemia supervened at much the same partial pressure of O_2 whether CO was present or not.

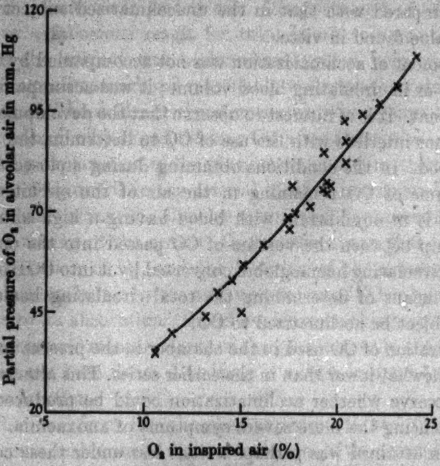

Fig. 5. Partial pressure of oxygen in alveolar air when air containing a varying percentage of oxygen is breathed.

A few closed-circuit experiments were made with a low proportion of O_2 in the circuit from the beginning; in one of these the alveolar O_2 pressure was found to be 44 mm. Hg, and the subject experienced symptoms of slight anoxaemia. The arterial O_2 pressure, however, calculated on the assumption of free diffusion of CO between alveolar air and blood, would have been 64 mm. Hg, a value which would not cause any symptoms of anoxaemia. This result suggests that the partial pressure of O_2 in the arterial blood was at least as low as that in the alveolar air.

DISCUSSION

The experiments described above confirm the previous finding that repeated exposure to an atmosphere containing low concentrations of CO induce some degree of acclimatization to CO. The evidence is (*a*) diminution in symptoms with repeated exposure to the same atmospheric concentration of CO, (*b*) a lowering of the proportion of COHb in the blood when a steady state is attained as compared with that in the unacclimatized subject and with the equilibrium value found in vitro.

The development of acclimatization was not accompanied by any change in red-cell count, or in circulating blood volume; it was accompanied by a slight fall in Hb content. It is of interest to observe that the development of acclimatization does not interfere with the use of CO to determine the volume of the circulating blood. In the conditions obtaining during a closed-circuit experiment the volume of CO remaining in the air of the circuit must be very small, since it is in equilibrium with blood having a high affinity constant. The relationship between the volume of CO passed into the circuit, and the proportion of circulating haemoglobin converted by it into COHb can therefore be used as a means of determining the total circulating haemoglobin even though the subject be acclimatized to CO.

The concentration of CO used in the chamber in the present series of experiments was somewhat lower than in the earlier series. This alteration was made in order to observe whether acclimatization could be produced without the subject experiencing the more severe symptoms of anoxaemia. The degree of acclimatization attained was perhaps a little less under these conditions, but it was nevertheless quite definite.

It has already been demonstrated that no change occurs in the affinity constant of blood during acclimatization, and this too has been confirmed for the present series of experiments. Two alternative explanations must be considered: either the body develops the power of destroying CO after it has been absorbed, or less CO is actually absorbed after acclimatization has developed, i.e. some factor prevents the free diffusion of CO between alveolar air and blood.

The experiments with a closed breathing circuit were designed to test the first explanation; if CO is destroyed in the body, the inhalation of a measured volume of CO would produce a blood saturation lower than the calculated one. By comparing the blood saturation-time curves of some of the chamber experiments it is possible to obtain a rough estimate of the rate of destruction of CO that would yield the observed results. For example, the two experiments previously discussed, those performed on 7 December 1937 and 29 March 1938, both in a concentration of about 0·02 % CO (Fig. 3) may be considered: in the first, a final blood saturation of 27% COHb was reached; in the second, equilibrium was attained at 16·5% COHb.

If a tangent is drawn to the saturation-time curve of the first experiment it appears that at the moment when the blood was 16·5% saturated the saturation was increasing at a rate of 5·7%/hr. (Fig. 3). This rate of increase would represent the absorption of about 33 c.c. CO/hr. If the body were destroying CO at this rate it seems extremely improbable that the percentage of COHb in the blood could remain constant during a period of 40–60 min. rebreathing from the closed circuit, as in fact it did. Disappearance of CO in the body would also give a fallacious result for total blood volume, but the values obtained when the subject was acclimatized agreed very well with those obtained when unacclimatized and reasonably well with the values determined by the dye method. The evidence therefore seems to be against the hypothesis that the acclimatized subject can destroy CO after absorption.

The more likely explanation of the phenomena of acclimatization is that the partial pressure of O_2 or CO in the arterial blood is not dependent wholly on diffusion of the gases across the alveolar membrane. Two possibilities must be considered: the raising of the arterial O_2 pressure above that in the alveolar air by active secretion of O_2, and the lowering of the arterial CO pressure below that in the alveolar air by the active excretion of CO or by the imposition of some barrier to its absorption. Direct evidence on this point could only be obtained by tonometric measurements of the gas tensions in the arterial blood, and this has not yet been done. But there is a little indirect evidence that is worthy of consideration.

The data obtained from the chamber experiments and from the closed-circuit experiments enable one to make calculations of the arterial gas pressures; for example the arterial O_2 pressure can be calculated from equation (1), if it is assumed that p.p. CO (arterial blood) is the same as the partial pressure of CO in the air breathed, after due time has been allowed for equilibrium to be established. Similarly the arterial pressure of CO can be calculated if it is assumed that p.p. O_2 (arterial blood) is the same as the partial pressure of O_2 in the alveolar air. Figs. 6 and 7 show the results of these calculations for the experiments in the chamber. Assuming free diffusion of O_2 across the alveolar membrane, we obtain a curve for the difference between arterial and alveolar pressures of CO that is fairly regular in form, with this difference increasing pari passu with other evidences of acclimatization. If we plot the alveolar and arterial pressures of O_2, calculated on the assumption of free diffusion of CO across the alveolar membrane, we obtain a curve for the difference in pressure (see Fig. 7) that is less regular in form. It shows a peak during an experiment with an atmospheric concentration of only 0·02% CO. On the hypothesis that the alveolar membrane secretes O_2 it is difficult to explain this increase in secretion when the degree of anoxaemia was small.

During some of the closed-circuit experiments the O_2 in the circuit accidentally fell low enough to cause symptoms of acute anoxaemia (tremor, dizziness

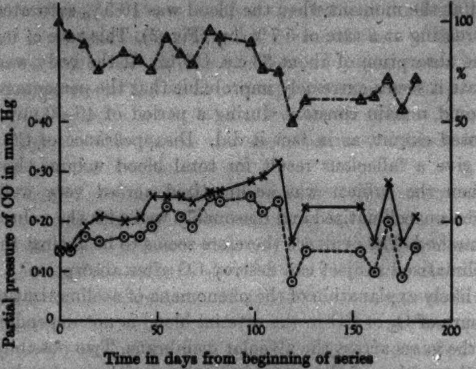

Fig. 6. Series of exposures to constant concentration of CO. Partial pressure of CO: ×—× in the atmospheric (and alveolar) air, as determined by analysis; ⊙---⊙ in the arterial blood (calculated); △———△ partial pressure in arterial blood as percentage of partial pressure in alveolar air.

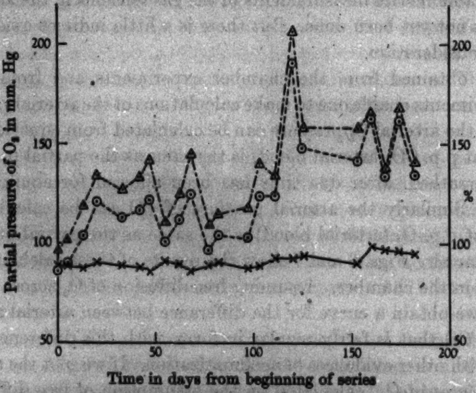

Fig. 7. Series of exposures to constant concentration of CO. Partial pressure of O_2: ×—× in the alveolar air as determined by analysis; ⊙---⊙ in the arterial blood (calculated); △———△ partial pressure in arterial blood as percentage of partial pressure in alveolar air.

and on a few occasions loss of consciousness). When the subject's blood contained 25–30 % COHb these symptoms appeared when the alveolar O_2 fell to about 6%, but were absent when the alveolar O_2 was at a normal level. The symptoms appeared at about the same alveolar O_2 pressure whether the subject was acclimatized or not, although on the theory that acclimatization involves the secretion of O_2 the arterial O_2 pressure would have been over 9% of an atmosphere in the acclimatized subject. The subject lost consciousness when the alveolar O_2 fell to 4–5%, and in one experiment the acclimatized subject became unconscious when the alveolar O_2 fell to this level although the calculated arterial O_2 was 7·5% of an atmosphere.

This evidence is not conclusive, but these few pieces of indirect evidence are in favour of the hypothesis that the lungs excrete or prevent the diffusion of CO, and against the hypothesis of O_2 secretion.

This hypothesis of the active exclusion of CO is attractive because it offers a possible explanation of the discrepancy between estimations of the partial pressure of O_2 in arterial blood made by different methods. The indirect method of determining arterial O_2 pressure, employing the inhalation of CO, would yield reliable results only in the non-acclimatized subject. The repeated use of this method with the same subject might produce some degree of acclimatization to CO, and thereby render it unsuitable for determining arterial O_2 pressure.

SUMMARY

1. A single human subject was repeatedly exposed to low concentrations of CO in an airtight chamber; each exposure was prolonged until the COHb% attained a steady value.

2. The degree of acclimatization was indicated by (*a*) the diminution in severity of the symptoms during successive exposures to the same concentration of CO, (*b*) the discrepancy between the observed COHb% at the end of an exposure, and the COHb% obtained in vitro when the subject's blood was equilibrated with a mixture containing O_2 and CO at the same partial pressure as in the alveolar air.

3. Acclimatization was not accompanied by changes in the red-cell count, in the proportion of reticulocytes, or in the blood volume.

4. The results of inhaling a measured volume of CO from a closed-breathing circuit confirmed the existence of acclimatization, and demonstrated that no appreciable destruction of CO occurred in the body.

5. The value of the constant for the partition of Hb between O_2 and CO, as determined in vitro, remained unaltered as acclimatization developed.

6. A possible explanation of these results is discussed.

This work was carried out in the Departments of Mining Research and of Industrial Hygiene and Medicine, University of Birmingham. Thanks are due to Prof. Moss for his permission to use the airtight chamber, and to the staff of his Department for their co-operation.

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THE ACTION OF TETANUS TOXIN ON THE RABBIT'S IRIS

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As a result of his investigation of the peripheral action of tetanus toxin, Harvey (1939) suggested that the condition of 'local tetanus' is caused by a disturbance of the normal processes of cholinergic transmission at the neuromuscular junction of skeletal muscle. The effect of this toxin on smooth muscles innervated by cholinergic fibres does not appear to have been studied hitherto. We have begun such an investigation by observing the effects on the muscles of the iris that result from the injection of a small quantity of tetanus toxin into the anterior chamber of the eye. This has proved a satisfactory method of localizing the effect of the toxin, and has shown that this substance exerts a paralysing action on the cholinergic fibres to the sphincter pupillae.

MATERIALS AND METHODS

Dried preparations of ammonium sulphate-precipitated toxin were made from cultures of the CN655 strain of *Cl. tetani* by the method described by Ipsen (1941*a*). The powdered toxin was kept in a vacuum desiccator, and fresh solutions of it in sterile 0·9% NaCl were made shortly before each inoculation. No solution was more than ½ hr. old at the time that it was injected. The toxicity of the dried preparation was repeatedly tested by intravenous injection into mice and showed little deterioration over the period of the experiments. The LD$_{50}$ of the dried toxin, calculated from Ipsen's (1941*b*) Table 83, was 0·075 μg. The powder contained a small amount of $(NH_4)_2SO_4$ as an impurity.

Before inoculation, the rabbits were lightly anaesthetized with 'veterinary nembutal' (0·75–1·5 c.c. intravenously), and, to ensure local insensibility, 2 drops of 5% cocaine-HCl were instilled into the conjunctival sac. When the cornea became anaesthetic, the eyeball was immobilized as much as possible by pressing on it from below with one finger placed on the medial half of the lower eyelid—the pressure being applied dorso-laterally towards the roof of the orbit. The carefully sharpened needle (size 26) of a tuberculin syringe was then introduced into the anterior chamber through the cornea just in front of the sclero-corneal junction at the inner or outer canthus (see Fig. 2, 2), and 0·05 c.c. of the solution was injected. The amount of toxin varied from 0·25 to 200 μg., but in most of the experiments a standard dose of 25 μg. was adopted.

In control experiments, the same volume of one of the following solutions was injected into the opposite eye: (1) toxin solution of the same concentration, in which the toxin had been neutralized by the previous addition of an amount of antitoxin (Wellcome tetanus antitoxin globulins:

1000 i.u./c.c.) which was estimated to represent about fifty times the immunological equivalent of the toxin; (2) toxin solution of the same concentration but previously mixed with a potent *Cl. botulinum* Type A antitoxin (containing less than 1/100 i.u. of tetanus antitoxin)—it was estimated that in the final mixture any tetanus antitoxin present would neutralize less than $\frac{1}{5000}$th of the toxin present; (3) the usual toxin solution boiled for 5 min.; (4) 0·9% NaCl solution.

In the experiments upon the sympathetically denervated iris, the superior cervical ganglion was removed aseptically on both sides in two rabbits. The structures removed were examined histologically and identified as sympathetic ganglion. An interval of 9 and 10 days respectively was allowed for the degeneration of the post-ganglionic fibres to the iris, before proceeding to inoculate the eye with toxin.

The inoculated eyes were observed at intervals, and the pupillary diameter was measured (to the nearest mm.) on both sides with a ruler placed just in front of the eye. This measurement was carried out for the two sides under identical conditions in diffuse daylight, and was recorded without correction for the error introduced by the refractive power of the cornea. The reaction to a sudden illumination with a 60 W. lamp at 7 cm. distance from the eye, was also measured on both sides. In addition, a number of photographic records of pupils was obtained by a method essentially the same as that described by Thomson (1947), to whom we are indebted for the loan of the necessary apparatus. This consisted of a Cine-Kodak special camera fitted with a device for synchronizing electrically the flash from a photo-flood lamp with the opening of the camera shutter; this flash was produced by the opening of an electromagnetic shutter fitted to the front of a light-tight box containing the photo-flood lamp (750 W.), and lasted 0·01 sec. By this means, photographs were taken of the iris before it had had time to react to the light. The rabbit was placed in a rabbit-box and its head held immobile at a standard distance from the lens. For the purpose of subsequent measurement, a millimetre scale was also photographed at the same distance. The pupils were photographed in each experiment (i) in diffuse light; (ii) after a period of dark-adaptation, which, owing to the difficulty of keeping the animal still for a longer period, was standardized at 3 min.; and (iii) after a 30-sec. period of intense illumination with two 100 W. lamps with reflectors at a standard distance (25 or 50 cm.). For the photographic studies, albino rabbits were used.

In the experiments on electrical stimulation of the nerves supplying the iris, the rabbits were anaesthetized with nembutal and the cervical sympathetic nerves were exposed on both sides. The skull was trephined and its bony vault removed with bone forceps. Haemorrhage was controlled by diathermic coagulation of bleeding points. The cerebral hemispheres were scooped out, exposing the optic nerves which were cut anteriorly to the chiasma. The intracranial portion of each oculomotor nerve was identified on the roof of the cavernous sinus.

In these last experiments the diameter of the pupil was measured on both sides with calipers. Measurements were taken before and after anaesthesia, and before and after faradic stimulation of equal intensity and duration of the cervical sympathetic and oculomotor nerves on both the intoxicated and the control sides.

RESULTS

Nature of pupillary changes produced by the toxin

Mydriatic effect. Within 24–48 hr. of the inoculation, a change was observed in the size of the pupil on the side injected with active toxin. In diffuse daylight the pupil was visibly larger than before the inoculation and more open than that on the control side. Ruler measurements made on the two sides are listed in Table 1. Similar differences in size were obtained from measurements of the photographs of the two eyes after they had become dark adapted.

This mydriatic effect was once produced with as small a dose of toxin as 0·25 μg.; we failed, however, to obtain it in two other animals with this dose and later in the same animals with 2·5 μg. It was produced with unfailing regularity in ten rabbits by a dose of 25 μg. Other rabbits inoculated with 50 μg. or more also showed the same pupillary changes. In none of these animals did any sign of tetanus appear either in the muscles of the orbit or elsewhere; the effect of the toxin appeared to be entirely localized to the eye. In a few instances the inoculation gave rise to a small patch of turbidity in the aqueous humour of the anterior chamber. This cleared up after 5 or 6 days and was not the cause of the paralysis observed, since the paralysis developed in the absence of any turbidity, and in those in which turbidity did appear, the mydriatic effect persisted long after the opacity had disappeared.

TABLE 1. Changes in pupillary diameter in twelve rabbits, produced by intra-ocular tetanus toxin (ruler measurements)

Dose of toxin (μg.)	Control eye	Time of measurement (days after inoculation)	Vertical diameter of the pupil in diffuse light (mm.)	
			Control side	Intoxicated side
0·25	Uninjected	7	5	10
25	Uninjected	3	7	11
		7	8	12
25	Uninjected	3	5	9
		6	6	10
		13	7	10
		17	6	9
		20	7	7
25	Same amount of toxin boiled for 1 min.	1	4	10
		5	5	10
		30	6	10
25	25 μg. tetanus toxin + excess of tetanus antitoxin	10	5	11
25	25 μg. tetanus toxin + *Cl. botulinum* antitoxin	5	10	10
		11	10	9
		18	6	10
25	25 μg. tetanus toxin + *Cl. botulinum* antitoxin	1	10	10
		5	12	12
		12	12	12
		15	10	12
25*	Uninjected	8	8	10
50*	Uninjected	1	8	8
		3	7	10
		8	6	10
		15	7	9
50	0·1 c.c. of sterile 0·9% saline	1	8	10
		5	8	10
50	Uninjected	3	5	11
		6	7	11
		14	7	11
		17	6	10
50	Uninjected	1	4	8
		3	6	11
		8	5	11

* Both superior cervical ganglia excised 9 and 14 days previously.

The pupillary changes were usually obvious within 48 hr. of inoculation: in several instances they began within 24 hr. The mydriasis lasted for 2½–5 weeks.

Reaction of the iris to light. For 24–48 hr. after the mydriatic effect had developed, weak reactions to light were sometimes obtained, but by the fourth day this response had disappeared (Pl. 1, fig. 1). At this more advanced stage, ruler measurements taken before and during bright illumination (60 W. lamp at 7 cm.) showed a complete absence of the ipselateral light reflex on the inoculated side, whereas it was present in every case in the control eye. The more accurate photographic measurements, which confirm this absence of reaction to light, are listed in Table 2. In these experiments, the eye was illuminated with a 200 W. lamp at 50 cm. distance for a period of 30 sec. before the photographs were taken. More intense illumination with a 750 W. photoflood lamp at 13 cm. for 5 sec. was equally ineffective. The absence of light reflex did not appear to be due to any failure of light perception on the part of the retina, because when the inoculated eye was approached with some object or subjected to intense illumination, powerful nictitation of the three eyelids was observed.

TABLE 2. Absence of reaction to light in tetanus intoxicated eyes (photographic measurements)

		Horizontal pupillary diameter (mm.)			
		Control side		Intoxicated side	
Dose of toxin (μg.)	Time of measurement (days after inoculation)	Dark adapted (3 min.)	Light adapted (30 sec.)	Dark adapted (3 min.)	Light adapted (30 sec.)
25	4	6·5	3	9	9
25	6	9	6	10	10
25*	10	5	3·5	11	11
50	3	7	4	10	9·5

* This rabbit received the same dose of toxin, neutralized by an excess of tetanus antitoxin, in the control eye.

Specificity of the reaction

The following control experiments were performed to demonstrate that these pupillary effects were produced specifically by the tetanus toxin:

(i) *Experiments with tetanus antitoxin.* Three rabbits were inoculated in the left eye with 25 μg. of toxin, and in the right eye with the same amount of toxin which had been neutralized just previously by a gross excess of tetanus antitoxin. In all three animals, the typical syndrome developed in the left eye, but the right was unaffected, the pupil showing no change in diameter and the iris reacting to light in the usual manner.

(ii) *Effect of a non-specific serum* (Cl. botulinum *antitoxin*). That the protective action of tetanus antitoxin is not due to a non-specific effect of the serum was shown in two experiments in which it was replaced in the mixture with the toxin, by a batch of *Cl. botulinum* Type A antitoxin which was practically free from tetanus antitoxin. This antitoxin was chosen because the paralytic symptoms produced by tetanus toxin bear a certain resemblance to those seen in botulism, and it was, therefore, of interest to see whether they could be prevented by *Cl. botulinum* antitoxin. It was found that this antitoxin was devoid of any protective action since the tetanus toxin produced a mydriasis and loss of reaction to light as severe in its presence as without.

(iii) *Other controls.* In two other experiments, the control eye was injected with 0·05 c.c. of toxin solution (25 μg.) which had been boiled for 5 min., and with the same volume of sterile 0·9% NaCl solution, respectively. In neither of these controls did the responses typical of the intoxication appear.

Effect of the toxin on the iris after sympathetic denervation

To exclude the possibility that the dilator effect of the toxin was due to the overaction of the sympathetic nerve fibres and endings in the radial muscle of the iris, experiments were performed on two rabbits in which the superior cervical ganglion on both sides had been excised 9 and 10 days previously. Injection of the standard dose of toxin was followed by the usual mydriatic effect in these eyes, and this change was also associated with the usual loss of response to light.

Effect of acetylcholine

To test the contractility of the sphincter pupillae in the intoxicated eyes, four of the inoculated animals were anaesthetized with nembutal, and 0·05 c.c. of a sterile solution of acetylcholine-HCl dissolved in 0·9% saline was injected into the anterior chamber on the affected side. In the first experiment the dose of acetylcholine-HCl was 20 μg., but in the others it was 1 μg. In each instance there was a powerful constriction of the pupil (Pl. 1, fig. 2), within 10–15 sec. of the injection. In the last experiment (on a rabbit at the 30th day of the intoxication) the duration of this acetylcholine effect was compared with the effect on the normal side. It was prolonged on the intoxicated side and a slight myosis was still present 24 hr. after the injection.

Effect of eserine

A myosis could also be produced in the intoxicated eyes by instilling eserine into the conjunctival sac (Pl. 1, fig. 3). A 1% solution of eserine sulphate dissolved in 0·9% saline was used at first and myotic effects were obtained with 1–2 drops (0·06–0·12 c.c.) in four rabbits. The first noticeable change in the pupil (a reduction in diameter of 1 mm.) occurred after 5–6 min. and the change was complete within 10 min., the final diameter of the pupil being 2–3 mm.

To compare the time course of this myosis with that of the normal eye, experiments were made in which the eserine was instilled simultaneously into the conjunctival sacs on both sides, and measurements made of the two pupils at intervals subsequently. With fresh 1% eserine, the myotic effect was so powerful that there was little difference between the control and intoxicated eyes, although the onset of the myosis appeared slightly earlier in the former. In later experiments weaker solutions of eserine (0·5, 0·1 and 0·01%) were used, and, with these, the myotic effect of eserine was often weaker on the intoxicated side, and was also slower in its development and sometimes in onset.

Effect of stimulating the oculomotor and cervical sympathetic nerves

On the induction of anaesthesia before the exposure of the nerves for stimulation, it was observed that the pupil of the intoxicated eye underwent contraction, while that of the control eye dilated, the diameters of the two becoming approximately equal. It was from this intermediate equilibrium position (see Langworthy & Ortega, 1943) that the effects of the subsequent brief faradic stimulation (5–10 sec.) of the oculomotor and cervical sympathetic nerves were determined. The immediate changes in pupil diameter which resulted from this excitation are shown in Table 3. After these measurements had been made, eserine was introduced into the intoxicated eye, either by the instillation of 2–5 drops of a 1:1000 solution of eserine sulphate, or by the intraocular injection of 100 μg. of the drug. On repeating, after a few minutes, the stimulation of the oculomotor nerve to the intoxicated eye, small constrictions of the pupil occurred even when the paralysis had formerly been total; the pupils of the three animals showed contractions of 1·5, 1·0 and 1·0 mm. respectively.

TABLE 3. Changes in the diameters of pupils (averages of three to six determinations) after stimulation (of equal strength and duration) of the oculomotor and cervical sympathetic nerves on the control and intoxicated sides

Dose of toxin (μg.)	Days since inoculation	Nerve stimulated	Changes in pupil diameter (mm.)	
			Control side	Intoxicated side
100	6	Cervical sympathetic	+2·0*	+2·0*
		3rd	−4·3	−0·2
100	19	Cervical sympathetic	+3·7	+2·5
		3rd	−1·6	nil
50	12	Cervical sympathetic	+2·3	+2·0
		3rd	−3·8	−0·5

* Single observations only.

These experiments show that tetanus toxin has little or no effect on the adrenergic nerve fibres which supply the dilator muscle of the iris. On the other hand, the cholinergic nerve fibres to the sphincter pupillae from the oculomotor nerve, appear to be almost completely paralysed by the toxin, though an improvement takes place in the presence of eserine.

DISCUSSION

The injection of small quantities of an active preparation of tetanus toxin into the anterior chamber of a rabbit's eye results in a slowly developing and persistent mydriasis. This effect might result either from an over-action of the dilator muscle of the iris (or of the adrenergic nerve fibres to it), or from a paralysis of the sphincter pupillae (or of its cholinergic nerve supply). It seems unlikely that the former explanation is correct because the pupillary response to light is lost simultaneously, and also because the mydriasis occurs in its typical form in animals in which the previous removal of the superior cervical ganglion has ensured the degeneration of the post-ganglionic sympathetic nerve fibres. Indeed, sympathetic denervation does not materially affect the development of this mydriasis, either by the interruption of inflowing nervous impulses, or by the sensitization of the dilator muscle fibres to humoral influences. It appears more likely that the pupillary dilatation and the absence of reaction to light, result from a paralysis of the more powerful sphincter pupillae, which in the albino rabbit is the muscle principally concerned with the response of the iris to light (Langworthy & Ortega, 1943). However, since this muscle still contracts promptly and powerfully when a small quantity of acetylcholine is injected into the aqueous humour, it is evident that the injurious effect of the toxin is not falling primarily on the muscle fibres themselves. Coupled with this fact, the absence of a normal response to oculomotor nerve stimulation, and its partial restoration in the presence of eserine would suggest that the paralysis results from a disturbance of the humoral transmission at the cholinergic nerve-endings in the sphincter. The paralysis from tetanus toxin thus differs radically from that produced by atropine, for, unlike atropine, the toxin does not appear to produce a block between the transmitter and the muscle fibres.

Stimulation of the cervical sympathetic nerves shows that the function of the adrenergic fibres is little affected by the toxin. From this fact, together with the augmentation by eserine of the myosis produced by oculomotor nerve stimulation, it may also be inferred that the toxin does not interfere so much with the conduction of nervous impulses as with humoral transmission.

The paralytic effect of tetanus toxin in this nerve-muscle system may be compared with that described by Harvey (1939) in voluntary muscles in local tetanus. He observed that despite the persistence of local spasticity the tension response to a maximal motor-nerve volley became progressively reduced as the intoxication developed, and after 2–3 weeks it sometimes disappeared entirely, although direct stimulation of the muscle fibres either electrically or by the intra-arterial injection of acetylcholine produced a contraction that was at least as great as normal. In his discussion, Harvey suggested that the toxin may bring about its paralytic effects by causing an abnormal leakage from, and eventually a depletion of, the acetylcholine depots at the cholinergic nerve endings, and compared this state of the nerve-endings with that found during nerve degeneration. This associated paralysis which is present in experimentally produced local tetanus is in line with the occurrence of paralytic forms of tetanus which have been observed clinically in man (see Courtois-Suffit & Giroux, 1918), particularly in patients with cephalic tetanus, who may exhibit ophthalmoplegia and paralysis of the lingual and of the whole or part of the facial nerves.

There are minor clinical features of tetanus, usually thrown into the background by the more striking skeletal muscular disturbances, which suggest that the activity of the other parasympathetic nerves in the body may be similarly deranged in this intoxication. Retention of urine is of common occurrence, and manometric determinations of intravesical pressure made by Eastman & Nesbit (1942) on a patient with tetanus, have shown that the bladder may be completely atonic. Tachycardia, which cannot be accounted for by concurrent pyrexia, has been described in a series of patients suffering from tetanus by Dean (1917). Clinically, also, constipation is often a troublesome feature of this disease, and we have observed a complete absence of peristaltic movements and retention of faeces in the intestines of tetanus-intoxicated mice when the viscera have been examined immediately after death. There is no suggestion from these clinical data, or from our own experiments, that the onset of paralysis in these parasympathetic nerves is preceded, as in the voluntary motor nerves, by a phase of spasticity. Thus, we have not seen any sign of myosis at any time before the onset of the pupillo-motor paralysis. The comparison between the effects of tetanus toxin and those of nerve degeneration is again applicable here, for when a parasympathetic nerve to smooth muscle is cut, there is no phenomenon in the muscle comparable to the fibrillation seen after motor-nerve section. The difference may be due to the absence of end-plates in the muscle systems concerned.

SUMMARY

1. The injection of tetanus toxin into the anterior chamber of the rabbit's eye results in a dilatation of the pupil and a loss of reaction to light. This effect starts within 1–2 days of the injection and lasts 2½–5 weeks.

2. The reaction is specific and is prevented by tetanus antitoxin, but not by the antitoxin to *Cl. botulinum* Type A.

3. The effect is produced in the sympathetically denervated iris.

4. Under anaesthesia the pupillary dilatation on the intoxicated side is reduced. Stimulation of the cervical sympathetic nerve then produces dilatation of the pupil on the intoxicated as well as on the control side. On the other hand, stimulation of the oculomotor nerve has little or no effect on the pupil of the toxin-injected eye, except after eserine when real myotic responses were observed.

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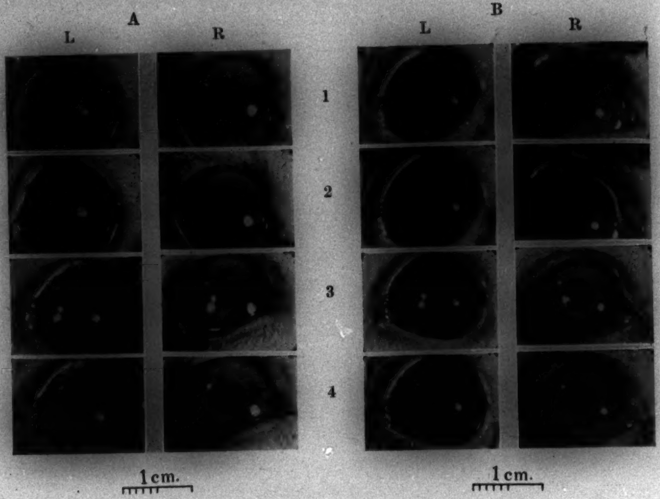
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Fig. 1.

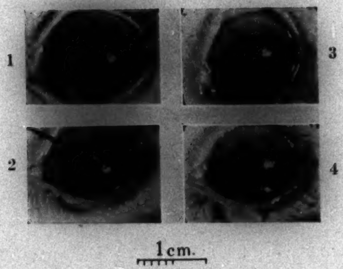

Fig. 2.

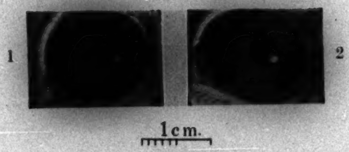

Fig. 3

To face p. 53

5. The mydriasis was not due to a paralysis of the muscle fibres of the sphincter pupillae, because this muscle can still respond vigorously to acetylcholine. The results suggest that tetanus toxin does not affect the smooth muscle or the adrenergic nerve fibres in the iris, but that it paralyses specifically the cholinergic nerve-endings of the oculomotor nerve.

We are grateful to the Director of the Wellcome Physiological Research Laboratories for providing us with the strain of *Cl. tetani* used for preparing the toxin, and for the gift of botulinus antitoxin. Most of these experiments were performed in the Department of Pathology, Guy's Hospital Medical School, but a few were continued by one of us (N.A.) at the Department of Physiology, University College, London; some of the expenses of this research were covered by a grant to him from the Ella Sachs Plotz Foundation.

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EXPLANATION OF PLATE 1

Fig. 1. Successive photographs of the pupil showing the mydriasis and loss of reaction to light which is produced by tetanus toxin. Two rabbits were injected with toxin in the left eye (50 μg. in A, 25 μg. in B) the right eye serving as a control. Photographs were taken of both eyes on the 3rd (in A) and the 4th (in B) days of the intoxication. 1. The pupils in diffuse light. 2. After 3 min. dark adaptation. 3. Effect of 30 sec. intense illumination (200 W. at 50 cm. distance). 4. Illumination just ended. Calibration in mm. The white spots are due to reflexion of lamps.

Fig. 2. Successive photographs of a rabbit's eye which was injected with 50 μg. of tetanus toxin 13 days previously, showing the response of the iris to an intraocular injection of acetylcholine. 1. Initial size of the pupil (10·5 mm. horizontal diameter). 2. The syringe needle is introduced into the eye and its point is seen in the anterior chamber; 1 μg. of acetylcholine HCl was injected at this moment and the needle was withdrawn. 3. 30 sec. later; the diameter of the pupil is now 6 mm. 4. 90 sec. after the injection (pupil 5 mm.).

Fig. 3. Myotic effect of eserine in an intoxicated eye (25 μg. of toxin injected 10 days previously. 1. Initial size of the pupil (10 mm.). 2. After eserine, 1 drop of 0·5% eserine sulphate was instilled into the conjunctival sac 20 min. before this photograph was taken. Pupil 4 mm.

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RODS, CONES AND THE LOCALIZATION OF PRE-EXCITATORY INHIBITION IN THE MAMMALIAN RETINA

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The single spike record from a retinal element or unit, isolated by the micro-electrode technique, provides us with valuable information about the message delivered by the ganglion cells of the retina to the higher visual centres. The study of such records has already made it possible not only to detect excitation and inhibition in the retina but also to analyse the sensitivities of the actual receptors to different spectral wave-lengths (see Summary, Granit, 1947).

This paper is concerned with the relationship between the amounts of excitation and inhibition produced at the threshold of stimulation for the receptors of the cat retina as well as with how these two processes are related to the reactions of the rods and cones. Such differences as we have found in the association of excitation and inhibition with rod and cone reactions can probably be related to the manner in which their synaptic connexions are made.

THE PROBLEM

The present problem may be best understood with the aid of Fig. 1. In the diagram on the right of the figure three single retinal nerve fibres (whose reactions to stimulation are picked up by the micro-electrode) are illustrated, one transmitting entirely from rods, and the other two from both rods and cones. Such a convergence of retinal end-organs has been demonstrated histologically by Polyak (1941) and physiologically by Granit (Summary, 1947) in several different animals. In the cat, most if not all of these receptor units (i.e. receptors, bipolar and ganglion cells) contain some rods (Granit, 1945), and this fact has been utilized in the work to be discussed here.

If the discharge of such a retinal element simply represented the responses of the receptors, the result of illumination would be a more or less *pure on-effect*. In practice, however, the majority of the retinal elements which we have isolated responded to stimulation with both on- and off-effects, and some even with *pure off-effects*. This must mean that pre-excitatory inhibition (Granit, 1947) can act in some part of the complex retinal element suggested in Fig. 1 and, with more attention to detail, in Fig. 8.

Our immediate object was to discover the relationship between the sensitivity of different retinal elements to the onset and cessation of illumination and whether this relationship was the same for elements containing rods only as for those containing both rods and cones. In order to separate the reactions of the rods and cones, even when they were connected to the same nerve fibre, we compared the responses to two wave-lengths, a blue of 0·460 μ. and a red of 0·650 μ. Since the observations were all made on fully dark-adapted cats (the animals remained undisturbed in the dark box for not less than 1 hr. after the micro-electrode was in position and before the observations were begun) in which the retinal responses are dominated by visual purple, blue is a far more effective stimulus than red. In order to detect any responses which were *not* due to visual purple the intensities of blue and red could theoretically be adjusted so that they become equally effective as bleachers of visual purple; any inequality in the response would then probably be due to other retinal mechanisms.

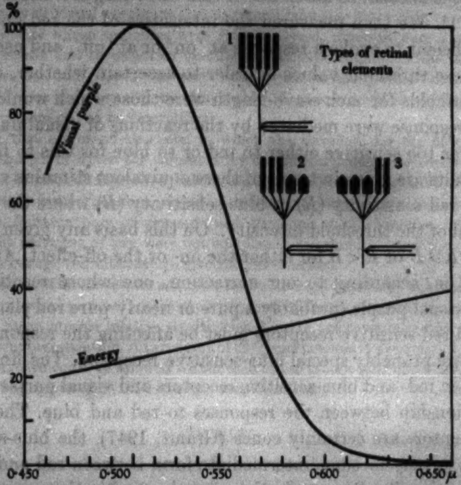

Fig. 1. Absorption curve of visual purple and schematic illustration of energy output of colorimeter lamp (straight line).

Top right-hand corner: diagram of types of retinal element postulated for the cat with the micro-electrode in position on the nerve fibre. 1, pure visual purple (rods only); 2 and 3, mixed rods and red-sensitive receptors (cones).

Our actual procedure was to calculate a correction factor for each of the two wave-lengths of our spectrum chosen (0·650 μ. and 0·460 μ.), based on their absorption by visual purple (Lythgoe, 1937), the energy distribution of the lamp used to produce the spectrum, and on the quantum intensity of the spectrum. These correction factors were adjusted so that the factor for a green wave-length of 0·500 μ. (the peak of the visual purple absorption curve) should be equal to unity. When such corrections are applied, the red wave-length is found to have only about 0·0045 % of the bleaching power for visual purple of the blue. This is because so much less of this wave-length is absorbed by visual purple (Fig. 1). We then measured the intensities of the red and blue which were just sufficient to evoke a response at 'on' or at 'off', and used the factors to correct these threshold values in order to ascertain whether, for any given unit, the thresholds for each wave-length were those which would be expected if the unit's response were mediated by the reactions of visual purple alone, or whether it was too sensitive either to red or to blue for this to be the case.

All our results are given in terms of these equivalent stimulus values and are expressed as red-sensitivity (R) or blue-sensitivity (B) where the sensitivity is the reciprocal of the threshold intensity. On this basis any given element may show $R=B$, $R>B$ or $R<B$ for either the on- or the off-effect. An element for which $R=B$ is, according to our correction, one whose reactions are conditioned by visual purple (probably a pure or nearly pure rod element). Where $R>B$ special red-sensitive receptors must be affecting the response and where $R<B$ there are probably special blue-sensitive receptors. The final balance of power between red- and blue-sensitive receptors and visual purple will be given by the relationship between the responses to red and blue. The special red-sensitive receptors are certainly cones (Granit, 1947), the blue-sensitive ones may be rods or may be some intermediate form between rod and cone. However, the situation $R<B$ is rare in the cat retina and in these experiments was only found among the pure on-elements.

It should also be possible by analysing the relation between the off- and on-components of a responding on-off-element to obtain information about the amount of inhibition occurring. We can assume that a high off/on sensitivity ratio, that is a lower threshold for the off- than for the on-effect (off > on), reflects an increase in inhibition, although the relationship between the ratio and the amount of inhibition need not be a direct one. By studying both the R/B ratio and the off/on ratio and their relationship to one another, we should learn more about the part played by the cones in the responses of the cat retina and also to what extent they are concerned with inhibition.

METHOD

The large Wright colorimeter (Wright, 1946) was set for the two wave-lengths chosen (0·650 μ. and 0·460 μ.) and re-calibrated in terms of energy output; special attention was, of course, given to the removal of stray light.

The micro-electrode technique has already been described in detail (Granit, 1945, 1946, 1947). Decerebrate cats were used; these were given 4–6 c.c. of 10% urethane, intraperitoneally, to prevent head and eye movements.

Altogether 164 satisfactory elements were isolated in this series of experiments but, for various reasons, complete measurements were only made on 100. Each threshold, determined for both 'on' and 'off' and both for red and for blue, is based on an average of fifteen observations. The actual number of observations necessary to determine any given threshold was dependent on the amount of spontaneous activity present.

The material. Of the 164 elements, 16% were pure on-elements, 5% pure off-elements, and 79% on-off-elements. An element was not considered to be pure 'on' or pure 'off' unless its type of response remained the same when it was stimulated with a wave-length of 0·500 μ. (position of maximum sensitivity of visual purple) at the maximum intensity obtainable on the colorimeter.

Of the 100 elements whose responses were fully analysed, nineteen were pure on-elements, eight pure off-elements and seventy-two on-off-elements. The slight increase in the combined percentage of pure on- and pure off-elements simply reflects the greater chance of making a complete analysis where there are only two thresholds (those to red and blue) instead of four (red and blue to both on and off) to be determined.

It was decided that six on-off-elements, in which the threshold for the off-component was extremely high, and six in which the threshold for the on-component was correspondingly high (only to be elicited by maximum green stimulation in each case) could be added to the pure on- and pure off-groups respectively. Therefore, judged by this less exacting criterion, there were twenty-five pure on-elements, fourteen pure off-elements and sixty-one on-off-elements. Of the sixty-one on-off-elements the reactions of two were not completely measured because the colorimeter did not deliver a high enough red intensity to give the red on-effect.

A statistical analysis of all the results was made by Dr Leonard Goldberg (Karolinska Institutet). All the results are given in terms of sensitivity (reciprocal of the threshold) so that greater relative values of R or B mean greater relative sensitivities. We are not concerned with absolute sensitivities in the present investigation. An accuracy of 20% was possible in determining each threshold so that a 40% variation could be allowed within each of the groups into which the elements were divided. Most of the results were clear without statistical analysis.

RESULTS

For all elements for which it could be obtained, the on-sensitivity to blue (B-on) was taken as the fundamental sensitivity and made equal to 100 in calculating the results. For those elements which showed no on-effect the off-sensitivity to blue (B-off) was used.

On-elements: $R=B$ in 44% of these, $R>B$ in 24%, and $R<B$ in 32%. Thus, the majority of these elements show a pure visual purple response while a third are blue-sensitive and only a quarter red-sensitive.

Off-elements: $R=B$ in 35·7%, $R>B$ in 64·3% and none had $R<B$. Therefore in this group two-thirds of the elements are red-sensitive, one-third are governed by visual purple and none are blue-sensitive.

On-off-elements: The main group is divided into two sub-groups, so that the sensitivities of the on- and off-responses can be examined separately.

(*a*) On-components: $R=B$ in 32·2%, $R>B$ in 67·8% and there were no blue-sensitive components. Thus, as for the pure off-elements, two-thirds of these elements were red-sensitive at 'on' and one-third were dominated by visual purple.

(*b*) Off-components: $R=B$ in 14·8%, $R>B$ in 85·2% and there were no blue-sensitive elements. Thus, in this sub-group most of the elements were red-sensitive, a small proportion only being dominated by visual purple.

TABLE 1

		On-off-elements						
	On-elements *R*-on/*B*-on	Group I: on>off (average off/on ratio=0·17)		Group II: on=off (average off/on ratio=1·02)		Group III: on<off (average off/on ratio=4·0)		Off-elements *R*-off/*B*-off
		On	Off	On	Off	On	Off	
R/*B* ratio	0·86	1·75	2·38	2·01	2·95	3·59	3·90	1·45

On account of the large range of variation the figures given for each group have been calculated from the logarithmic values.

The average values used in the figures and table are calculated from the logarithms of the actual figures. In the table the average *R*/*B* ratios for the three types (pure 'on', 'on-off' and pure 'off') of elements are given. For $B=100$ the average value for R for pure on-elements is 86 (R/B ratio 0·86) and for pure off-elements 145 (R/B ratio 1·45). These values are statistically significant. There is, therefore, a slight dominance of blue-sensitivity in the population of pure on-elements and a more marked dominance of red-sensitivity in the population of pure off-elements. Only one R/B ratio in the latter group fell below 1·0 (it was 0·73) and the maximum value was 2·53.

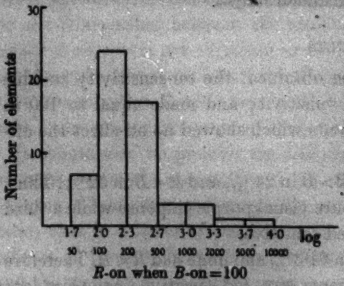

Fig. 2. Distribution of red-sensitivity for the on-components of on-off-elements.

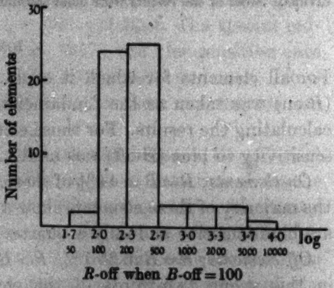

Fig. 3. Distribution of red-sensitivity for the off-components of on-off-elements.

For the on-off-elements the average values are of less interest on account of the wide individual variations ranging from $R/B=1·0$ to $R/B=50$. The results are, therefore, best illustrated by the distribution curves given in Figs. 2 and 3. These show the number of elements exhibiting various amounts of red-sensitivity for both the on-response (Fig. 2) and the off-response (Fig. 3). It will be seen that the really high R/B ratios are to be found among the on-off-elements.

However, the most striking point about the reactions of the on-off-elements is the enormous range of variation in the off/on ratio both for blue and for red. We shall come back to this point later.

If the on-off-elements are divided into three groups, according to whether off < on, off = on or off > on, then 47·9% of all the on-off-elements fall into the first group, 28·2% into the second and 23·9% into the third when the red wavelength was used as the stimulus. Almost the same percentages were obtained with blue. The majority of the on-off-elements are, therefore, more sensitive to the onset than to the cessation of illumination.

It can be seen from the table that there is a systematic increase in red-sensitivity as one passes from the pure on-elements by way of the off < on and off = on to the off > on of the on-off-elements. This means that, at least for the pure on- and on-off-elements, an increased sensitivity at 'off', which indicates an increased sensitivity to inhibition, is correlated with an increased sensitivity to red, and this indicates a more prominent cone reaction. It is true that the pure off-elements do not fit into this pattern since they show a lower R/B ratio than any of the on-off-elements. The table also shows that, within each of the three groups of on-off-elements, the R/B ratio, which can be used as a cone/rod index, is always higher for the off- than for the on-component.

General comments. Only on-off-elements were isolated in numbers large enough for further statistical analysis. We shall, therefore, summarize the results of a general comparison of the properties of the three types of element before going on to consider the reactions of the on-off-elements in more detail.

We have seen that in a population of pure on-elements the majority show either $R=B$ or $R<B$. This means that the on-elements are largely dominated by rods. One would expect a retina containing a high proportion of rod-elements to have a fairly simple synaptic organization in which the on-response of the receptors, integrated by the bipolar and ganglion cells, would be more or less directly reflected in the discharges recorded from the nerve fibres. The guinea-pig has such a retina (Granit, 1945) in which most, although not all, of the elements show no off-effect. Histologically the guinea-pig retina contains rods only.

Further, the dominator curve of the guinea-pig eye is always the scotopic one (reflecting the dominating influence of visual purple) irrespective of the state of adaptation; there is no Purkinje shift (Granit, 1942). However, after light-adaptation the shape of the sensitivity curves indicated that some blue and green modulator elements were present and on a few very rare occasions there was evidence of red modulator elements as well. The blue modulator elements were quite easy to detect in this pure rod retina and this finding becomes particularly interesting in view of the present indication of blue-sensitive receptors among the pure on-elements of the cat retina. We have here additional evidence in favour of the idea that the blue-sensitive end-organs of the retina are, in fact, rods (cf. Willmer, 1946). The reactions of both blue- and green-sensitive receptors might be due to photochemical substances produced by simple transformations of the visual purple molecule as a result of light-adaptation. There is also a possibility that the properties of those elements whose sensitivity to red is but little greater than that to blue are due to the presence of green- rather than red-sensitive receptors. The sensitivity curve of the green modulator unit extends farther towards the red end of the spectrum than that of the blue one and might, therefore, produce a final slight preponderance of red- over blue-sensitivity.

In general, one may say that the cone/rod indices (R/B ratios) found by us for the different types of element indicate cone activity in those elements which are subject to inhibition (show an off-effect). That this is not the whole story is shown by the fact that a third of the pure off-elements gave a pure rod response ($R=B$) and a quarter of the pure on-elements gave a cone response ($R>B$). In these cases we have rods associated with inhibition and cones free from inhibition (these may possibly be intermediate forms between the true rod and the true cone). The values of the R/B ratios given in the table suggest that the on-off-elements cannot be made up by the combination of a pure on- with a pure off-element. Rather must they represent various combinations of four types of receptor, inhibitory (off) cone, excitatory (on) cone, inhibitory (off) rod, and excitatory (on) rod. The fact that the on- and off-components of an on-off-element have different R/B ratios is impossible to understand if one assumes that, within a given element, a cone discharging down an inhibitory (off) path is identical with one discharging down an excitatory (on) path. However, the excitatory cones in one element might, as we shall see later, be inhibitory for *another* element.

The on-off-element seems to be the really important one in the cat retina, a retina which has, according to Walls (1942), 'a very respectable number of cones...about a third as many as we ourselves'. This type of element accounts for about 80% of the retinal units and of this number about 66%, if one judges by the behaviour of the on-component, or about 86%, if one judges by the behaviour of the off-component, contain cones. It is clear that the on-off-element must be the most important one not only for colour discrimination but also for other visual discriminations. For instance, the mechanism for appreciating contrast must be located in a retinal structure capable of responding both at 'on' and at 'off'.

The spectral sensitivity curve of the dark-adapted cat (Granit, 1945) shows too that cones must participate in the response to stimulation by yellow and red light. The average sensitivity to this part of the spectrum was too high to be mediated by a pure visual purple mechanism and higher than was found in the guinea-pig. These particular measurements were all made on the on-effect irrespective of the type of element under investigation.

Analysis of the on-off-elements. The fact that the red- and blue-sensitivities may vary independently of one another shows that the two types of stimulus must often affect different structures within the same element. This, of course, is the basis of our idea that, provided the *R*/*B* ratio is greater than unity, the red-sensitivity affords a measure of the amount of cone activity, and therefore perhaps the number of cones, within the element in question.

The distribution curve of the off/on sensitivity ratio for red is given in Fig. 4. Although the extreme values are rare there is a wide variation in the values found for this relationship. We shall discuss the significance of this variation, which appears to be a fundamental property of the retina, later on.

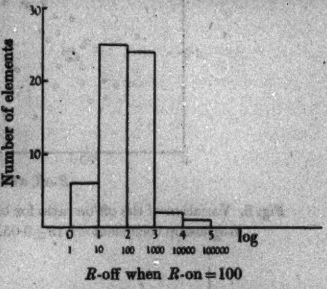

Fig. 4. Distribution of off/on ratio to red.

In addition there is a close correlation between *R*-off and *B*-off (see Figs. 5 and 6). Individual elements may behave differently but, in general, a high sensitivity to red at 'off' is associated with a high sensitivity to blue at 'off' and vice versa. This suggests that the reactions of the red- and blue-sensitive receptors within the element are combined before they reach the ganglion cell, and are responsible for a common off/on ratio. In other words, the off/on ratio is determined at the synapses.

On the other hand, there is, as we have already seen, a tendency for the *R*/*B* ratio to increase with the off/on ratio (Fig. 7). When the *R*/*B* ratio is greater than unity, threshold stimulation with red light is actually a selective cone stimulation since the stimulus must be below the threshold of the other receptors. Since the *R*/*B* ratio tends to be higher for those elements which are exceptionally sensitive to 'off', we have yet another association between the presence of cones and an off-effect.

No definite correlation could be found between the red-sensitivity at 'on' and at 'off'. However, it is perhaps worth mentioning that when the values were arranged according to the red on-sensitivity and then divided into two equal groups, all the higher off-sensitivities to red were in the group which contained the *R*/*B* ratios of over 1·75 for 'on'. This means that the on-components of elements highly sensitive to 'off' tend to show cone activity too, but the absence of precise correlations between the on- and off-thresholds forbids the drawing of definite conclusions.

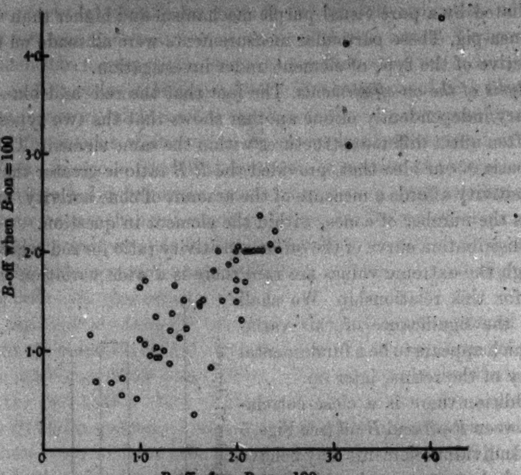

Fig. 5. Variation of the off/on ratio for blue with the off/on ratio for red (log. units). Regression coefficient = 1·19 ± 0·05. Correlation coefficient = 0·96 ± 0·01.

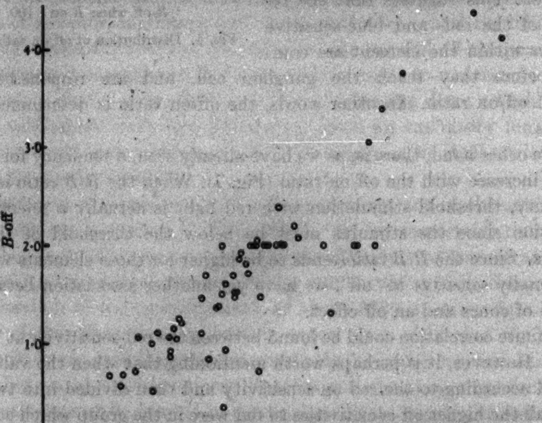

Fig. 6. Variation of *R*-off for *B*-off = 100 with the off/on ratio for blue (log. units). Regression coefficient = 0·82 ± 0·07. Correlation coefficient = 0·83 ± 0·04.

CONCLUSIONS

The only retina so far investigated which is quite without synapses is that of the horseshoe crab, *Limulus*. It is only possible to record on-responses from this eye (Hartline & Graham, 1932) so that it is likely that off-responses do not appear unless the retina contains synaptic structures. If the cones of the cat's retina were themselves capable of responding to stimulation by off-effects one would expect to find a close correlation between the off/on ratio for red and the *R*/*B* ratio for the off-effect. Instead the dominant correlation is between the off-sensitivities to red and to blue. As we have seen, this can only mean that the off-effect arises in the synapses and that it does not much matter which trigger, rod or cone, is touched off at the receptor level so long as the message passes through a cone synapse or is somehow affected by a mechanism usually associated with a cone response. At this point it should once more be emphasized that the use of threshold stimuli means that, where the *R*/*B* ratio is high, red stimulates cones only. Rods can only respond to a threshold red stimulus where $R=B$ or $R<B$.

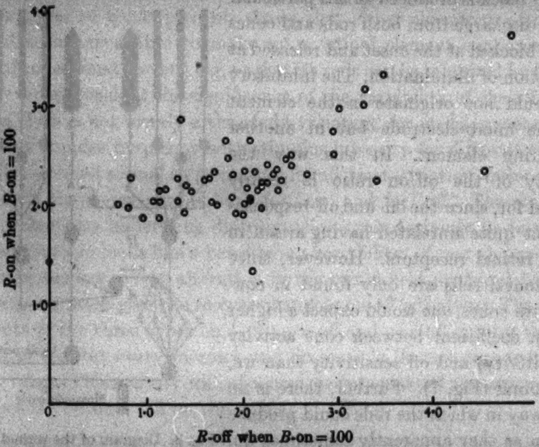

Fig. 7. Variation of the *R*/*B* ratio for the on-component with the off/on ratio (log. units). Regression coefficient = 0·29 ± 0·02. Correlation coefficient = 0·93 ± 0·02.

We can now formulate four criteria for the off-effect-inhibition system. It must be located between the receptors and the nerve fibre in the synaptic structures of the retina; it must in some way be closely connected with the cone synapses, but at the same time it must be possible for the rods to activate it; finally, it must have a design which can explain the wide variations found in the off/on ratio, variations which are quite outside the range to be expected from a relatively homogeneous biological system.

The straightforward synaptic connexions from the receptors to the ganglion cells by way of the bipolar cells do not fulfil these conditions very well. The differences between the rod and cone paths are not, so far as we know at present, great enough to explain why the R/B ratios for 'on' and 'off' in the same element are not identical. It is also difficult to see any reason for the variability of the off/on ratio.

Our four conditions are better satisfied if the horizontal cells, to which, indeed, Polyak (1941) has already attributed inhibitory properties, are included. He shows them as connecting pure cone pedicles to the vitreal ends of *both* rods and cones (Fig. 8). If we assume that an inhibitory block is produced at this particular spot, the discharge from both rods and cones would be blocked at the onset and released at the cessation of illumination. The inhibitory block would not originate in the element under the micro-electrode but in another neighbouring element. In this way the variability of the off/on ratio is readily accounted for, since the on- and off-responses are in fact quite unrelated having arisen in different retinal receptors. However, since the horizontal cells are only found in connexion with cones, one would expect a higher regression coefficient between cone activity (red-sensitivity) and off-sensitivity than we, in fact, found (Fig. 7). Further, there is no obvious way in which the rods could produce inhibition as they apparently sometimes do. This difficulty could perhaps be got over by assuming that some of the elements in which $R=B$ do contain cones but in numbers too small to change the reaction to $R>B$. If this were the case the apparent inhibitory action of some rods would in fact be due to these cones. When one considers the large number of retinal elements which show off-effects and the small number of cones relative to rods in the cat retina, it is clear that cones must be extremely efficient inhibitors of the reactions of adjacent elements whether these are due to rods or cones. If cones were unable to inhibit the responses of adjacent cones it would be very difficult to explain how selective stimulation with threshold red could produce any off-effect at all.

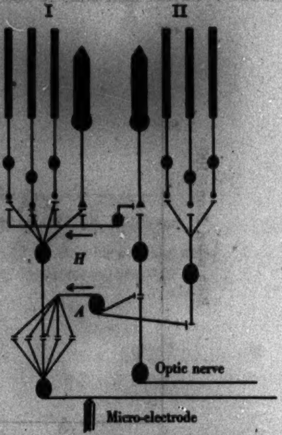

Fig. 8. Diagram of the retinal structure illustrating suggested inhibition pathways. *H*, horizontal cell path (Polyak); *A*, amacrine cell path.

However, inhibitory activity need not necessarily be restricted to the association paths of the horizontal cells. Unfortunately, we know far less about the amacrine cells. They are numerous, to be found in all parts of the retina and form association paths rather higher than the horizontal cells. Reference to Fig. 8 will show that all our conditions would be satisfied if the amacrine cells, either alone or in combination with the horizontal cells, caused the inhibitory block which must precede an off-effect. In particular, the third condition, that rods as well as cones could set off the reaction, would be fulfilled since the amacrine paths connect the retinal elements *after* the rod and cone responses have reached a common path. The variability of the off/on ratio is even easier to account for on this view than if the horizontal cells are the agents of inhibition, since the higher the level the greater the likelihood of differences in the reactions of adjacent elements.

The fact that the R/B ratio can show such big variations, in spite of the small *absolute* number of cones present, shows that either the group of receptors connected to a nerve fibre to make up a retinal element containing a fair number of cones must be very big, or that its rods can be inhibited by its cones.

Finally let us consider the significance of the variability of the off/on ratio for vision. This is not merely a threshold variation, for we have measured the variation of impulse frequency with stimulus intensity for different colours in twenty-five isolated retinal elements and found great variation in the shapes of the intensity-frequency curves both for 'on' and for 'off'. The clue to the retinal mechanisms underlying discrimination must certainly lie in what, on the surface, merely looks like a bewildering variability. Every change of light, every slight shadow, every alteration in the visual field produced by minute eye movements must give rise to ever-changing on-off pulsations. The presence of elements of the three types on > off, on = off and on < off ensures that these fluctuations register every increase and decrease in illumination over a wide intensity range. This activity will, of course, be very marked at the points where the images of contours fall on the retina.

SUMMARY

1. The micro-electrode technique was applied to the analysis of the reactions of 100 isolated retinal elements in the dark-adapted cat. Equivalent stimulus values for the dark-adapted eye for a red ($0{\cdot}650\,\mu$.) and a blue ($0{\cdot}460\,\mu$.) wavelength were obtained by applying a correction factor based on the sensitivity of visual purple and the energy distribution of the colorimeter lamp. If, after this correction has been applied, threshold sensitivity to red is equal to that for blue ($R = B$) then the reactions of the element in question must be dominated by visual purple. If $R > B$ then red-sensitive receptors (cones) must determine the reactions and if $R < B$ blue-sensitive receptors must be active in the element.

2. By measuring the R/B ratio for pure on- and pure off-elements, it has been possible to demonstrate that the pure on-elements are dominated by rods, and that cones are present in a great majority of both on-off-elements and pure off-elements. Blue-sensitivity was restricted to the pure on-elements.

3. The off/on ratio was measured for the on-off-elements. Since the occurrence of an off-effect is evidence of preceding inhibition the off/on ratio is a measure of inhibitory activity just as the *R*/*B* ratio is a measure of cone activity. It was found that the appearance of inhibition was associated with the presence of cones.

4. The results have made it possible to formulate four conditions which must be satisfied by any retinal structure postulated as responsible for the inhibitory system. These conditions are best satisfied by the amacrine cells possibly in combination with the horizontal cells.

5. The off/on ratio showed a variation of 1 to 100,000. The extent of this variation is considered to be of fundamental importance for visual discrimination. It must also be taken into account in any consideration of the localization of inhibition in the retina.

6. The *R*/*B* ratio for the on-off-elements varies from 1 to 50 despite the limited absolute number of cones, so that the retinal element must either contain a large number of receptors or the cones must be able to inhibit the rods within the same element.

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INSPIRATORY VASO-CONSTRICTION IN PATIENTS AFTER SPINAL INJURIES

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Observations on vaso-constriction in the finger after deep inspiration in normal subjects have been reported by Gilliatt (1947), in whose paper earlier work is also discussed. In contrast to previous authors, Gilliatt concluded that there is no satisfactory experimental evidence to indicate the nature of the afferent path in this reflex.

Whatever the mechanism involved, it is established from the results of earlier workers that a single deep inspiration causes vaso-constriction and a decrease in electrical resistance of the skin, similar to that resulting from various extrinsic sensory stimuli (Stürup, Bolton, Williams & Carmichael, 1935; Carmichael, Honeyman, Kolb & Stewart, 1941). A deep breath has also been reported to cause dilatation of the pupil (Somogyi, 1913).

The present paper describes the results of observations on patients after spinal injuries. Digital volume has been recorded in a series of patients with lesions of the spinal cord at various levels, and their vaso-motor responses to deep inspiration have been compared with those caused by extrinsic sensory stimuli, applied in each case above the level of spinal injury.

METHODS

Patients lay in bed in a warm darkened room, and finger and toe volumes were recorded optically with glass plethysmographs, as described by Stürup *et al.* (1935). It was not possible to calibrate membranes in situ, and so no quantitative estimate can be made of the degrees of vaso-constriction obtained. In addition, the sensitivity of the recording membranes varied slightly, as did the volume of the dead space of the recording system. However, it will be seen that in spite of differences in sensitivity of the membranes used, the appearance of reflex vaso-constriction in any one case is unmistakable, there being a characteristic fall in total volume and in the volume of each pulse.

In the simultaneous recording of the volume of two digits, it became important to eliminate the effects of membranes of different sensitivity. Accordingly, where such a difference existed, the volume changes of a single digit were recorded successively on both membranes during a single experiment.

In recording respiration, the stethograph was worn round the chest below the level of the nipple, and its position was varied somewhat to suit the comfort of each patient.

A sudden loud noise, pin-pricks on the face and neck, or ice applied to the same region were used as extrinsic sensory stimuli; all of these were observed by Stürup *et al.* (1935) to cause vaso-constriction of the skin. These stimuli were all applied to the face or neck, so that in no case was the spinal lesion high enough to interrupt the sensory pathway to the brain. Thus any effect of a lesion on the digital response to these stimuli was due to an interruption of efferent paths. They did in fact test the completeness of a lesion at a particular level.

RESULTS

Fourteen patients were examined, the levels of the lesions in the spinal cord varying between segments C6 and T11; of these, three have been omitted from discussion here, as the lesions were shown to be incomplete; one further case has been omitted, in which results were ambiguous, and where a re-examination was not possible. The results obtained with the remaining ten patients have been collected in Table 1. In each case, clinical tests of sensory and motor function indicated that there was complete loss of functional continuity of the cord at the appropriate level. Where stated in Table 1, areas of remaining sympathetic innervation were determined by a thermo-regulatory sweat test (using quinizarin) (Guttmann 1937). In addition, distension of the bladder was carried out with all but one of these patients and digital vaso-motor responses recorded optically, or by skin temperature measurement, as described by Guttmann & Whitteridge (1947). Only in the few cases specifically mentioned were the levels of lesions confirmed by operation.

TABLE 1

	Segmental level of lesion			Response to extrinsic stimuli		Response to deep inspiration		Response to bladder inflation	
Case	Right	Left	Sweat test	Finger	Toe	Finger	Toe	Finger	Toe
1	C6/7	C6/7	Not done	Nil	Nil	Vaso-constriction	Nil	Vaso-constriction	Vaso-constriction
2	T1/2	T1/2	Few pin-points of sweat on nose, lips, chin only	Nil	Nil	Vaso-constriction	Vaso-constriction	Vaso-constriction	Vaso-constriction
3	C8/T1	C8/T1	Not done	Nil	Nil	Vaso-constriction	Vaso-constriction	Vaso-constriction	Vaso-constriction
4	T3	T1	Few pin-points on right side of nose	Nil	Nil	Vaso-constriction	Nil	Vaso-constriction	Vaso-constriction
5	T4	T4	Not done	Poor vaso-constriction	Nil	Poor vaso-constriction	Vaso-constriction	Not done	Vaso-constriction
6	T5	T5	Level of lesion confirmed	Poor vaso-constriction	Nil	Poor vaso-constriction	Nil	Not done	Not done
7	T6/7	T6/7	Level of lesion confirmed	Vaso-constriction	Nil	Vaso-constriction	Nil	Vaso-dilatation	Vaso-constriction
8	T6/7	T6/7	Not done	Vaso-constriction	Nil	Vaso-constriction	Nil	Vaso-dilatation	Vaso-constriction
9	T8	T7	Level of lesion confirmed	Vaso-constriction	Nil	Vaso-constriction	Nil	Not done	Vaso-constriction
10	T10	T10/11	Level of lesion confirmed	Vaso-constriction	Nil	Vaso-constriction	Nil	Vaso-dilatation	Vaso-constriction

The vital capacity after spinal injury

It was observed by Gilliatt (1947) that a normal subject breathing abdominally, with the chest bound, suffered a large reduction in vital capacity, and a much larger reduction in the volume which could be inspired from the normal expiratory position. However, patients after spinal cord injuries were found to have compensated for the paralysis of intercostal musculature to a considerable extent, by the use of the muscles of the neck and shoulders. By pulling the sternum upwards with the sterno-mastoid and other auxiliary inspiratory muscles, a patient with a lesion at the level of segment C6 was able to increase the antero-posterior diameter of the chest.

Results from two patients are given below:

Segmental level of lesion	Vital capacity	Max. volume inspired from normal expiratory position
	(l.)	(l.)
C6	2·8	2·2
T6	3·4	2·6

(Measured while sitting in wheeled chair.)

From this it was concluded that even in patients with high spinal cord injuries, sufficient air could be taken into the chest to provide the necessary afferent stimulus for skin vaso-constriction.

Vaso-motor responses

The vaso-constriction which follows deep inspiration or extrinsic sensory stimuli in normal subjects needs no detailed description here, as it has been dealt with fully by previous authors (Stürup *et al.* 1935). A typical record (Fig. 6) is included for comparison with the results obtained on paraplegic patients.

Three precautions in interpretation of records require mention:

(1) Involuntary movements and muscle spasm, particularly in the lower limbs, occurred to a varying extent in different patients. In general, they tended to be most vigorous after a deep breath, and in some patients made the recording of toe volume exceedingly difficult. It was essential that passive volume changes of a digit due to involuntary movement and true vaso-motor responses should not be confused. The two most important criteria, in judging whether a given volume change was due to a vaso-motor reaction, were that the total volume change should be accompanied by a change in pulse volume, and that both should return slowly to their resting value within 30–60 sec.

(2) On some occasions, an extrinsic stimulus caused the patient to take a sharp inspiration, and this latter was found to be sufficient to cause a reflex vaso-constriction.

(3) In addition, a deep breath might cause contraction of the bladder, and this stimulus has been shown by Guttmann & Whitteridge (1947) to cause vaso-constriction of the skin below the level of the lesion in a paraplegic patient. An example of toe vaso-constriction accompanying emptying of the bladder is shown in Fig. 5*b*.

For purposes of description, the ten patients may be grouped into two distinct categories, according to the level of spinal cord injury, and the nature of their vaso-motor responses.

Group 1.

In four patients the cord lesions were above the level at which the sympathetic outflow to the hand leaves the cord. Therefore none of these patients showed any response in finger or toe to extrinsic sensory stimuli. These patients differed, however, in their vaso-motor responses to deep inspiration; two of the four patients showed a marked vaso-constriction in the finger after a deep breath, but no response in the toe (Fig. 1). As an example, Case 1 is described below.

W. J. B., male, aged 21 *yr*. (Fig. 1). On 30 April 1944, sustained fracture-dislocation of vertebrae C5 and C6 following a somersault during physical training. Since then there had been a complete transverse spinal syndrome below segment C7 on both sides, with motor paralysis of both lower limbs, trunk, forearms, hands and fingers. The only muscle working in the wrist was the extensor carpi radialis longus. The paralysed muscles of the upper limbs showed marked atrophy. There was complete loss of sensory function below dermatome C7, and loss of control of bladder and bowels. The neurological signs are still unchanged.

The remaining two patients in Group 1 showed a vaso-constriction in both finger and toe in response to a deep breath (Fig. 2). As an example, Case 2 is described below.

J. D., male, aged 21 *yr*. (Fig. 2). On 29 October 1944 sustained gun-shot wound of his neck, resulting in a complete transverse lesion at segment T1–2. Motor paralysis involved the intrinsic muscles of both hands, especially abductor pollicis brevis and opponens, as well as the lower limbs and trunk. At operation on 30 October 1944, a metallic foreign body was removed from the spinal canal at the level of the 7th cervical vertebra. The spinal cord was found 'not completely severed'. The transverse spinal syndrome is still complete.

To summarize, Group 1 includes four patients with high spinal cord lesions, who had apparently no cerebral or medullary control of the sympathetic outflow to the limbs, yet all of whom showed a vaso-constrictor response in the fingers to deep inspiration. In two of these patients, there was also a vaso-constrictor response in the toes to inspiration.

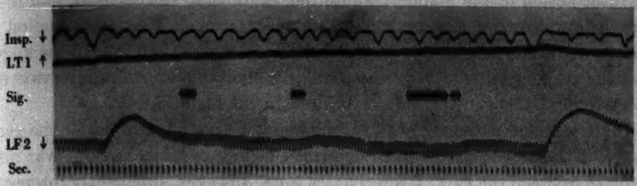

Fig. 1. Case 1. From left to right: responses to a deep inspiration, four pin-pricks on face and a further deep inspiration. From above downwards: *Insp.* ↓ =respiratory record inspiration downwards; *LT* 1 ↑ =left big toe volume, dilatation upwards; *Sig.*=signal for pin-pricks; *LF* 2 ↓ =left index finger volume, dilatation downwards. Finger vaso-constriction accompanies deep inspiration but not pin-pricks.

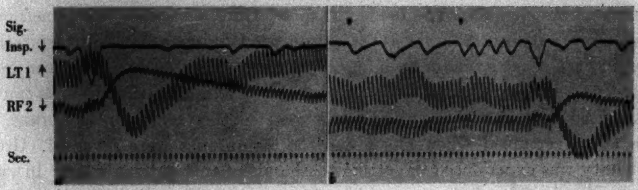

Fig. 2. Case 2. Two sections of record to show responses to (*a*) deep inspiration, and (*b*) two auditory stimuli, shown by signal, followed by deep inspiration. Symbols as in Fig. 1. Finger and toe vaso-constriction accompanies deep inspiration but not auditory stimuli.

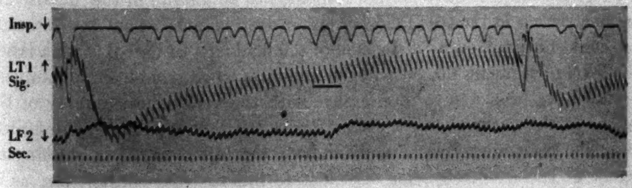

Fig. 3. Case 5. From left to right: responses to a deep inspiration, ice applied to face during signal and a further deep inspiration. The finger constricts poorly to both ice and deep inspiration while the toe responds only to deep inspiration. Symbols as in Fig. 1.

Although Head & Riddoch (1917) showed that deep inspiration could cause detrusor action of the bladder, we do not think that vaso-constriction in the toes in our cases was secondary to detrusor action. The latent period for vaso-constriction in the toes was not detectably longer than that in the fingers, and the response in the toes was regularly elicitable at short intervals irrespective of changes in the volume of fluid in the bladder.

There was one patient (Case 5) who cannot be included in either the group above or the second group to be described below, as his behaviour was intermediate between the two. His responses were, however, sufficiently interesting to justify a separate description.

A. H., male, aged 24 *yr.* (Fig. 3). On 9 March 1945 involved in a plane crash and sustained compression fracture of 3rd thoracic vertebra, resulting in complete transverse spinal syndrome at segment T4. Lumbar puncture revealed complete block and 400 mg. protein, so laminectomy was carried out by Mr Pennybacker at the Radcliffe Infirmary, Oxford. The spinal cord was found to be necrotic at the level of T4. The transverse spinal syndrome is still complete. As might be expected from the level of the lesion, a vaso-constrictor response to extrinsic stimuli was present only in the finger, but deep inspiration caused vaso-constriction in both fingers and toes (Fig. 3). It can be seen from Fig. 3 that in this case, the finger response to all stimuli was poor. This can be attributed only partly to the low sensitivity of the recording membrane, as the effect remained when finger and toe membranes were reversed. Great difficulty was experienced in getting the hands of this patient warm; in view of the level of his lesion, some interference with the efferent sympathetic supply to the hand would be expected, and may have been responsible for this.

Group 2.

This group includes all the patients with mid-thoracic or lower thoracic lesions. These subjects gave normal responses to all stimuli in the fingers. No vaso-constriction in the toes followed either deep breaths or extrinsic stimuli, but it could be obtained by distension of the bladder. In Case 6, with a lesion at the level of segment T5, poor finger responses to all stimuli were obtained; this may have been due to interference with the sympathetic supply of the hand, as in the patient with a lesion at the 4th thoracic segment described above. The remaining four patients showed the full response in the fingers, a threat or gesture often being sufficient to initiate vaso-constriction, as in normal subjects. Cases 9 and 10, whose records are shown in Figs. 4 and 5 respectively, are described below.

L. S., male, aged 26 *yr.* (Fig. 4). On 20 September 1943 blown up by a bomb and sustained fracture of the 6th, 7th and 8th thoracic vertebrae, resulting in complete transverse spinal syndrome at segment T7 on the left, and T8 on the right. Only the upper abdominal muscles were working, the middle and lower

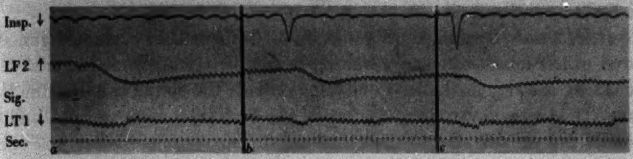

Fig. 4. Case 9. Three sections of record to show responses to (*a*) loud noise at signal (*b*) and (*c*) deep inspiration. Symbols as in Fig. 1. The records were taken with insensitive membranes. The finger constricts in (*a*), (*b*) and (*c*). The toe shows some involuntary movement but no vaso-constriction.

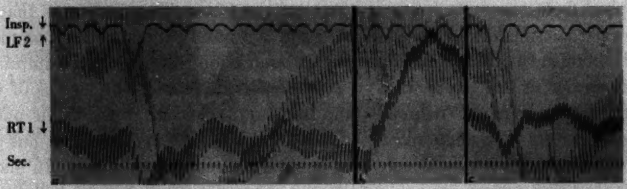

Fig. 5. Case 10. Three sections of record to show responses to (*a*) deep inspiration, (*b*) spontaneous contraction and emptying of the bladder, (*c*) deep inspiration. Symbols as in Fig. 1. Finger vaso-constriction follows deep inspiration but toe shows only passive dilatation due to movement of lower limb at time of breath. Toe vaso-constriction accompanies bladder emptying in (*b*). Very sensitive membranes used in this experiment (contrast Fig. 4).

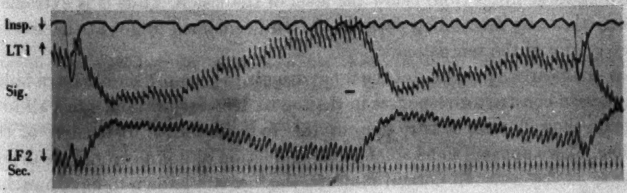

Fig. 6. Normal control. From left to right: a deep inspiration, pin-prick on face at signal and a further deep inspiration. Symbols as in Fig. 1. Both finger and toe constrict after deep inspiration and after pin-prick.

abdominals, as well as both lower limbs, were completely paralysed. Sensory loss was complete below the dermatome of T 7 on the left, and T 8 on the right. The lesion is still complete.

R. H. M., male, aged 21 *yr.* (Fig. 5). On 31 October 1944 sustained gun-shot wound of left lung and spine, resulting in complete transverse lesion at T 10. The X-ray showed the bullet to be lying outside the spinal canal at the level of the 11th thoracic vertebra, fracture of the 11th transverse process, and the articular process driven into the spinal canal. The complete transverse spinal syndrome at segment T 10 still persists.

DISCUSSION

The most interesting result of these observations has been the finding that, in some patients with a complete break in functional continuity of the spinal cord, vaso-constriction in response to deep inspiration may persist in digits supplied from segments below the level of the lesion. This indicates that in these patients inspiratory vaso-constriction is a purely spinal reflex, complete in the cord in the thoracic region. Such a conclusion rests on the assumption, made earlier in this paper, that in all the cases described, the lesions of the spinal cord were in fact complete, and that no descending sympathetic paths were left intact. The anatomical evidence for this is extremely meagre, but the clinical evidence does not seem to leave room for reasonable doubt. In many cases, tests of sensory and motor function were supplemented by a thermoregulatory sweat test, and complete anhydrosis below the level of the lesion was found in each case tested. Additional support is derived from the vasomotor responses of patients to extrinsic stimuli, such as pin-pricks on the face or loud noises. These unexpected stimuli tended to cause marked perturbation of the patient, accompanied by audible comment or a transient tachycardia, but in spite of this, vaso-constriction was uniformly absent in digits supplied by sympathetic fibres from below the level of the lesion. Thus it is extremely unlikely that any descending paths in the cord remained intact at the level of injury in any of the ten patients.

It is not clear why two out of the four patients in Group 1 should have failed to give vaso-constrictor responses in the toe to deep inspiration, since all four patients showed vaso-constriction in the toes following distension of the bladder. There appears to be a defect in conduction of unknown nature in this pathway in the cord. In normal subjects, the toes are known to have a rather greater tendency towards vaso-constriction than the fingers (Doupe, Robertson & Carmichael, 1937). No exact estimate can be made with regard to the level at which the afferent fibres concerned in this reflex enter the spinal cord. In the four Group 1 patients, the level of entry is presumably largely below segments C8–T1, while Case 5 suggests that some fibres enter below segment T4. From the fact that none of the patients with mid-thoracic and lower thoracic lesions show any toe vaso-constriction to deep breathing, it might be assumed that all the afferent fibres enter the cord above this level. Such an assumption is not justifiable, however, in view of the variability of the toe response in the Group 1 patients. The lower limit of entry cannot be defined in this way. All experimental results are, however, consistent with the general hypothesis that the afferent fibres concerned in inspiratory vaso-constriction enter the spinal cord mainly in the upper thoracic region, and that a reflex response can occur without the participation of higher centres.

SUMMARY

1. Vaso-motor responses to deep inspiration have been recorded in the fingers and toes of paraplegic patients.
2. In patients with a complete break in functional continuity of the spinal cord above the level of sympathetic outflow to the hands, inspiratory vaso-constrictor responses were still found in the fingers.
3. From this it is concluded that in these patients inspiratory vaso-constriction is a purely spinal reflex, taking place in the thoracic region of the isolated cord.
4. The responses of the toes to deep inspiration were more variable than those of the fingers; the reason for this is obscure.
5. All observations are consistent with the view that the afferent fibres concerned in this reflex enter the spinal cord mainly in the upper thoracic region, but the possibility that the limits of entry are somewhat wider than this is not excluded.

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VASO-CONSTRICTION IN THE FINGER AFTER DEEP INSPIRATION

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Reflex vaso-constriction of the digits after a deep breath has been reported by various investigators who have recorded finger volume (Goetz, 1935; Bolton, Carmichael & Stürup, 1936), or finger blood-flow (Wilkins, Doupe & Newman, 1938), and by those who have used photo-electric methods of recording peripheral blood-flow in man (Hertzman & Dillon, 1939). Mulinos & Shulman (1939) were able to observe directly a slowing or cessation of blood-flow through the capillary tufts of the nail-fold, after a single deep inspiration.

The most detailed observations on the origin of this reflex are those of Bolton, Carmichael & Stürup (1936), who find that vaso-constriction occurs after a deep thoracic inspiration, but not after an abdominal one; accordingly these writers suggest that the afferent endings may be situated in the chest wall, rather than in the thoracic viscera.

It was, however, established by the experiments of Lewis (1908), that the arterial blood pressure falls during thoracic inspiration, particularly during a deep inspiration, and that it does not fall, but rises, during abdominal inspiration, on account of the great rise in intra-abdominal pressure. Respiratory fluctuations in blood pressure have been investigated recently by direct arterial cannulation in man (Battro, Segura, Elicabe & Araya, 1944; Starr & Friedland, 1946), and a fall in systolic blood pressure of 15 mm. Hg during deep thoracic inspiration has been reported by the latter authors. Therefore, the first object of the present experiments was to ascertain whether the vaso-constriction which follows a deep breath is in fact initiated by a transient fall in blood pressure during the breath, and mediated by the carotid sinus or some other presso-receptor area.

For this purpose, systolic blood pressure and finger volume were recorded simultaneously, and later in the work, some other observations were made on thoracic and abdominal breathing, which will also be described.

METHODS

Finger volume was recorded optically by glass plethysmographs as described by Bolton, Carmichael & Stürup (1936). The volume of tissue in the plethysmograph varied between 10 and 18 c.c., and the size of plethysmograph was suited as far as possible to each subject.

Systolic blood pressure was recorded by a simple optical system, based on the method described by Evans & Mendelssohn (1946) (see their Fig. 2). A cuff round the wrist, fitted with a hand-bulb and aneroid, was connected with both sides of the sensitive membrane of a differential manometer. A mirror attached to the membrane reflected a beam of light on to a moving strip of bromide paper. The cuff was connected to the inside of the membrane by pressure tubing, while the connexion with the outside of the membrane was through a length of fine capillary tube. In this way slow pressure changes in the wrist cuff were transmitted to both sides of the membrane, while the fast changes occurring with each heart beat were damped out in the capillary tube, and reached one side of the membrane only. A wide-bore tube with a tap was placed in parallel with the capillary tube. The cuff could then be inflated rapidly, without a pressure gradient occurring across the membrane; when the tap was closed, a sensitive record of the pulsations in the cuff was obtained. The relation between cuff pressure and size of the pulsations of the membrane described by Evans & Mendelssohn (1946), was fully confirmed. For continuous recording of the blood pressure, however, a single cuff was not satisfactory. For this purpose the double-cuff system described by Evans & Mendelssohn was employed. An ordinary sphygmomanometer cuff was placed on the upper arm, and inflated to near systolic pressure; the wrist-cuff, inflated to 70–80 mm. Hg, served to record the volume changes in the lower arm, as blood passed under the upper cuff in systole.

The result of this arrangement is illustrated in Fig. 1; chest movements and the pressure in the upper cuff have been recorded simultaneously. In this record the fluctuations of systolic blood pressure are clearly shown. In addition, a quantitative reading of systolic pressure is obtained at any point on the record where it falls below the pressure in the upper cuff. In Fig. 1, for example, systolic pressure fell below 95 mm. Hg in inspiration, and rose above 105 mm. Hg during expiration.

One potential source of error in recording systolic pressure in this way was considered. It was possible that muscular movement in the upper arm might change the pressure under the upper cuff, and so cause the appearance of a false systolic pressure change on the record. At the suggestion of Dr K. Mendelssohn, a 2 l. bottle was connected with the upper cuff; in this way small volume changes of the upper arm were prevented from causing large pressure changes in the cuff. It can be seen in Fig. 1 that this pressure remained perfectly steady during the experiment.

Movement of the chest wall was recorded by a stethograph of corrugated tubing, at the level of the nipple, connected to a membrane of suitable sensitivity. For abdominal breathing, a broad webbing belt with a quick-release clip was worn below the stethograph.

When the volume of inspiration was to be recorded, the subject breathed from a spirometer through a Siebe-Gorman mouthpiece, with the nose clipped; mouth pressure was recorded from a T-piece inserted immediately below the inspiratory valve. For breathing air under pressure, this type of mouthpiece was not suitable; instead, the subject breathed through a short length of metal tubing, without valves, which was connected by a three-way tap to air under pressure in a Douglas bag loaded with weights. In this case, pressure in the mouth was recorded from a side tube in the mouthpiece, 1 in. from the lips. With this system, complete obstruction of the air inlet, or passive inflation of the lungs by air under pressure, could be carried out at any stage of the respiratory cycle, without intervention by the subject. Partial obstruction of inspiration and expiration was not attempted.

It was necessary to ensure that the subject was warm during an experiment. As a preliminary procedure, both arms were soaked in hot water for 10 min., after which the subject was wrapped in blankets upon a surgical couch, the back of which was raised at an angle of 45°. In addition, an electric blanket was used to warm the arm from which finger volume was taken. A darkened room and absolute quiet were essential. Under these conditions the finger vessels remained dilated and in a steady state for as long as 3 hr. In a few experiments, the subject sat upright in a chair; in all experiments, however, systolic pressure was measured at heart level, while finger volume was recorded from the opposite arm.

During the first experiment on any subject, finger constrictions of psychological origin occurred frequently in response to sounds, such as the motor of the camera, or the voice of the operator. Later, when the subject became sufficiently familiar with the technique, relatively complicated procedures could be carried out without any interference of this sort.

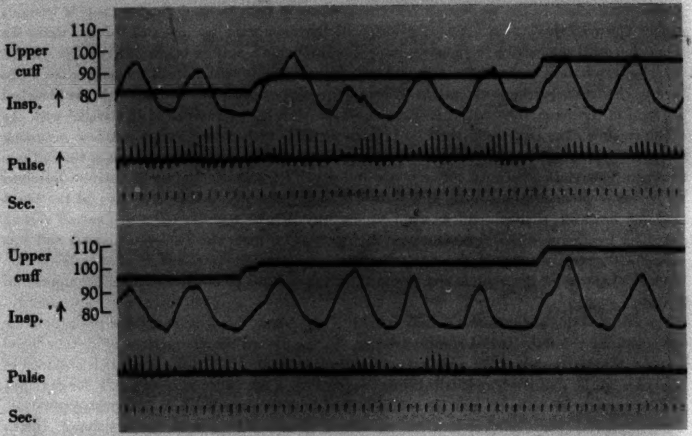

Fig. 1. The effect of deep thoracic breathing on systolic pressure. Symbols from above downwards: Upper cuff = pressure in upper arm cuff, in mm. Hg; Insp. ↑ = respiration, inspiration upwards; Pulse = pulsations at wrist; Sec. = time in sec. Systolic pressure is shown by the pulsations at the wrist, as recorded by the differential manometer during increasing pressures in the upper cuff. The two halves of the record are continuous.

RESULTS

In all, forty-two experiments were carried out on twelve healthy young adults. All subjects examined showed a diminution of finger volume, after a deep inspiration (Fig. 2*a*). The latency of the response was approximately 3 sec., but slightly longer latencies were observed when the finger was dilating at the time of the breath. A similar variation was observed by Downman, Goggio, McSwiney & Young (1943), in the paw-constrictor response of cats.

The normal response to a deep breath involved a diminution in volume of the finger of the order of 0·5–1·0%, while the volume of each pulse decreased to 50% or less of its former value. The fingers usually regained their previous volume within 60 sec.

The temperature of the inspired air

To ensure that inspiratory vaso-constriction was not related to the cooling effect of the inspired air, hot air saturated with water vapour was inspired from a suitable reservoir by two subjects. The temperature of the inspired air was measured by a thermocouple between the inspiratory and expiratory valves of the mouthpiece; in one experiment this temperature rose to 44·5° C. Neither in this nor in any other case was there a significant and reproducible diminution in the finger response (Fig. 2*b*).

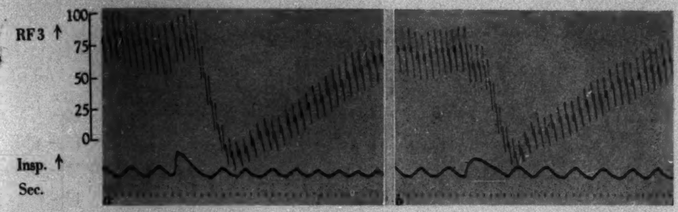

Fig. 2. The effect of breathing hot air, saturated with water vapour. Subject R. W. T. RF3 ↑ =volume of right 3rd finger (dilatation upwards). Volume scale in cu.mm. Other symbols as in Fig. 1. Two sections of a single record to show: (*a*) air inspired at room temperature 22° C.; (*b*) air inspired at 38° C. Total volume of finger in plethysmograph, 18 c.c.

This confirms the observations of Bolton, Carmichael & Stürup (1936) and of Mulinos & Shulman (1939) that inspiratory vaso-constriction of the finger is not due to cooling of the respiratory mucosa.

The composition of the inspired air

In view of the results of Bolton, Carmichael & Stürup (1936) and of Bolton, Carmichael & Williams (1936), it seemed unlikely that vaso-constriction after a single deep breath could be due to alteration in the gas content of the lungs. However, to ensure that the afferent stimulus was not related to dilution of alveolar CO_2 by a deep inspiration, a control experiment was performed in which deep breaths were taken from a bag containing 5% CO_2. There was no diminution in the size of the finger response when a deep breath of 5% CO_2 mixture was taken instead of air.

Systolic pressure and finger volume

Systolic pressure and finger volume were recorded simultaneously in seven subjects.

Normal inspiration. During a deep inspiration, systolic pressure fell in all subjects by 5–15 mm. Hg (Fig. 3*a*); this transient fall was followed by a rise in systolic pressure, lasting for three or four heart beats during the subsequent expiration, after which it returned to its resting level. Sometimes it showed a second slight fall of longer duration. The cause of this second fall in systolic pressure has not been further investigated. It occurs in spite of vaso-constriction of the fingers, and may be due to reduction in heart output, owing to alteration of the normal respiratory rhythm. Its appearance or non-appearance does not alter appreciably the diminution in volume of the finger.

Positive pressure inflation. Inflation of the chest with air under pressure was carried out in four subjects. Under these conditions a fall in systolic pressure during expansion of the chest and lungs did not occur, but was replaced by a small rise. In spite of this, typical constrictions of the finger were obtained in all four subjects (Fig. 3*b*). These occurred both when the positive pressure was of short duration, and when it was sufficiently long-lasting to cause a later fall in systolic pressure.

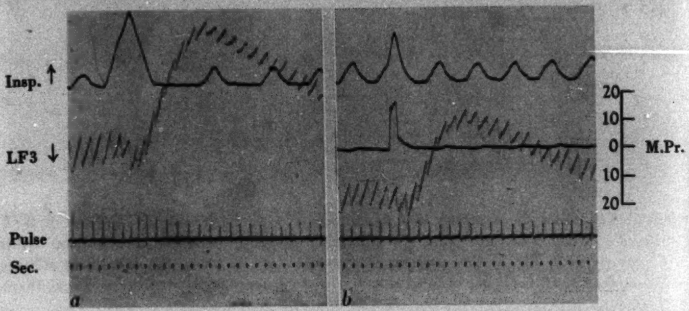

Fig. 3. Effect of (*a*) normal deep inspiration, (*b*) passive inflation with air under positive pressure. Subject D. W. M.Pr. = pressure in mouth in cm. H_2O; Pulse = pulsations at wrist, when upper cuff pressure = 95 mm. Hg, lower cuff pressure = 75 mm. Hg. Resting systolic pressure by auscultation, 105 mm. Hg.

Obstructed inspiration. In order to examine further the relation between systolic pressure and finger volume, the effect of an obstructed inspiratory effort was investigated in four subjects. The first time that the air inlet was suddenly obstructed, a subject usually gave a vaso-constrictor response. This was apparently due to the mental agitation involved, since, with practice, all four subjects were able to make strong inspiratory efforts against complete obstruction, without any vaso-constriction of the finger. For example, in the experiment illustrated in Fig. 4*a*, the pressure in the mouth fell to 20 cm. H_2O below atmospheric, and systolic pressure dropped markedly without any significant effect on the finger.

When the subject took a deep inspiration immediately after the release of the obstruction (Fig. 4*b*), finger constriction occurred after the inspiration, although this breath did not cause a fall in systolic pressure. Finger constriction also occurred when the chest was inflated with air under pressure, immediately after an obstructed inspiration (Fig. 4*c*), a transient rise in systolic pressure accompanying the inflation. Both in this and in the previous experiment where positive pressure inflation was used, it is assumed that the small rise in systolic pressure which accompanies the inflation is due to the expression of blood from the pulmonary vascular bed into the left side of the heart, by the increased air pressure. Starr & Friedland (1946) observed an increased output of the left ventricle, during positive pressure inflation in man.

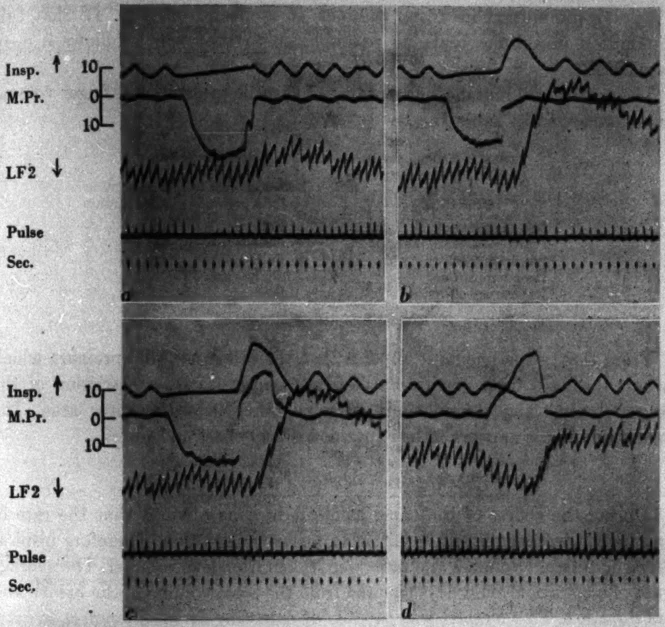

Fig. 4. Effect of obstruction of inspiration and expiration. Subject V. P. W. Symbols as in Fig. 3. Sections of a single record to show: (*a*) obstructed inspiratory effort; (*b*) the same followed by full inspiration; (*c*) the same followed by positive pressure inflation; (*d*) obstructed expiratory effort. Upper cuff pressure = 90 mm. Hg; lower cuff pressure = 75 mm. Hg. Resting systolic pressure by auscultation, 102 mm. Hg. *Note.* Systolic pressure falls below 90 mm. Hg in Fig. 4*a*.

Deep and obstructed expiration. The effects of deep and obstructed expiration were examined in five subjects. Deep expiration without obstruction never produced a vaso-constriction in any experiment. An expiratory effort against obstruction caused a rise in systolic pressure followed by a fall (Fig. 4*d*). With vigorous expiratory efforts, this fall in systolic pressure was sufficient to cause a passive diminution in finger volume. It is clear from Fig. 4*d* that this diminution in volume bears no resemblance to that which follows a deep breath.

The results of simultaneous recording of systolic pressure and finger volume are summarized in Table 1.

TABLE 1. Vaso-constriction and blood pressure changes

Procedure	Initial change in systolic pressure	Finger vaso-constriction
Deep inspiration	Fall	+
Positive pressure inflation	Rise	+
*Inspiratory effort	Fall	–
Deep expiration	Slight rise	–
*Expiratory effort	Rise	–

* Against complete obstruction.

From these experiments it appears that the fall in systolic pressure which occurs during a deep inspiration does not cause a vaso-constriction of the finger through the carotid sinus or some other presso-receptor area. The significant factor appears to be expansion of the chest and lungs.

The rate and volume of inspiration

During the course of the experiments it became obvious that the rate of inspiration was significant in initiating finger constriction. Therefore inspirations of different volumes and rates were taken from a spirometer. The average rate of inspiration could be calculated from the total volume of the breath and its duration. This method was capable of detecting large differences in rate, but where small volumes at fast rates were concerned, it was less accurate; the error involved in measuring average rate was increased with fast rates, and in these circumstances the expression provided no information as to variations in rate during the breath, which might be critical for vaso-constriction. For this reason it could only be considered as a rough guide.

It was found that small inspirations, taken at a fast rate, consistently caused finger constriction, while similar inspirations at a slower rate failed to do so. At slow rates, large inspirations were required to obtain a positive response. In all cases, inspiration was started from the normal expiratory position.

The results of three experiments have been collected; in Fig. 5 are shown the combinations of volume (V) and average rate (R) which were tested and whether or not the response occurred. The diagram indicates that when both V and R were small no vaso-constrictor response occurred, that when either was large (unless the other was extremely small) the response occurred, and that in an intermediate region the proportion of responses increased as either V or R increased.

There was no sharply defined threshold separating combinations of V and R giving the response from those giving no response, but instead a *probability* of response ranging from practical certainty under some conditions to zero under others. As an aid to fuller understanding of the relationship between breathing and constriction, it seemed desirable to investigate 'lines of constant probability', the curves connecting all pairs of values of V and R for which the probability of obtaining a response at any one trial has a specified value. Since only thirty-nine sets of observations were available, these curves could not be estimated very precisely, but statistical techniques enable some reasonable approximations to be obtained. A full account of the statistical analysis will be published elsewhere, and only an outline is given here.

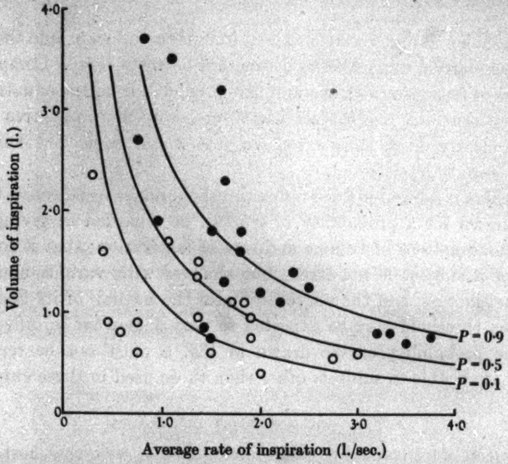

Fig. 5. Effect on finger volume of variations in volume and rate of inspiration in three normal subjects. ● = finger vaso-constriction; ○ = no finger response. For explanation see text.

Preliminary examination of the data suggested that the occurrence of a response was determined primarily by the magnitude of VR, the product of volume and rate, in such a way that the probability was constant at all points of a curve

$$VR = \text{constant},$$

a hyperbola, the constant depending upon the probability. In the statistical analysis, a slightly more general form of curve,

$$VR^{\beta} = \text{constant},$$

was adopted, β being a parameter which had to be estimated from the data. By means of the probit transformation, in the form adapted for use with individual records, a regression plane was fitted which represented the relationship between the transformed probability and the logarithms of V and R (Bliss, 1938; Finney, 1943, 1947). This analysis led to curves

$$VR^{0{\cdot}89} = \text{constant}$$

as the lines of constant probability, values of the constant being, for example, 1·10, 1·71 and 2·66 for probabilities of 0·1, 0·5 and 0·9 respectively (V being measured in litres and R in l./sec.). These curves are shown in Fig. 5; that for the 0·5 probability level gives estimated combinations of volume and rate for which, in repeated trials, the response is as likely as not to occur.

The fitted equation, however, is only an *estimate* of the true law of dependence of the probability on V and R. For each of the curves in Fig. 5, additional pairs of curves may be constructed to represent the probable limits of error; these bound a region within which the *true* curve is almost certain to lie, with a selected level of fiducial probability. For the 0·5 constant probability line, this region is narrow at the middle of the range, but broadens rapidly towards the extremes. For the 0·1 and 0·9 lines, the region is much wider on the side away from the 0·5 line than on the near side.

The data shown in Fig. 5 were obtained from three subjects, and the analysis that has been carried out made no distinction between them. Comparison of the numbers of responses with the numbers predicted from the equation shows no indication that any one subject was appreciably more sensitive than the others, and clearly much more extensive data would be needed if individual differences were to be detected.

Though there is no well-defined reflex threshold in the usual sense, the curve in Fig. 5 drawn for a probability of 0·5 can be regarded as giving median threshold combinations of volume and rate of inspiration; that is to say, the threshold of a subject is not fixed, but changes with various uncontrolled experimental factors, and the curve estimates the median of its fluctuations. As the reflex threshold may be expected to vary somewhat in different individuals, the probability curves drawn in Fig. 5 must not be regarded as necessarily applicable to subjects other than those used in these experiments.

Thoracic and abdominal inspiration

Four subjects were trained to respire abdominally, with the chest strapped in the expiratory position; after some practice they were able to take a deep abdominal inspiration, with negligible movement of the chest wall. Under these conditions, the volume which could be inspired from the normal expiratory position was found to be very small. For example, while the vital capacity of the subject R.W.G. was reduced from 5·2 to 2·8 l., the maximum volume which could be inspired from the normal expiratory position was reduced from 3·2 to 0·9 l. Thus the reduction in vital capacity of 2·4 l. was almost entirely at the expense of the complemental air. The effect of abdominal breathing was therefore further investigated with inspirations taken at fast rates.

In all four subjects, vaso-constrictor responses in the finger were obtained in this way. These responses were strictly comparable with those obtained when thoracic inspiration was restricted voluntarily to a similar volume. In the experiment illustrated in Fig. 6, finger volume was recorded continuously during a series of abdominal breaths, release of the chest strap, and a further series of thoracic breaths. The record shows that vaso-constriction occurred with a 900 c.c. inspiration when the breathing was abdominal, and with a 1200 c.c. inspiration when it was thoracic. Vaso-constriction was poor or absent with an abdominal inspiration of 700 c.c. and with a thoracic inspiration of 800 c.c.

By the technique used for these experiments, it has not been possible to detect any consistent and reproducible difference in the effects of thoracic and abdominal breathing on the volume of the finger.

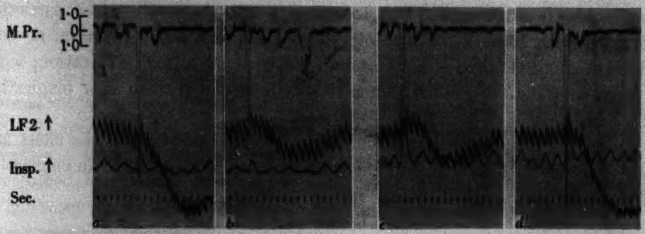

Fig. 6. Effect of thoracic and abdominal inspiration. Subject R. W. G. Symbols as in Fig. 3. Sections of a single record to show: (*a*) an abdominal inspiration of 900 c.c.; (*b*) an abdominal inspiration of 700 c.c.; (*c*) a thoracic inspiration of 800 c.c.; (*d*) a thoracic inspiration of 1200 c.c. Chest-strap released between (*b*) and (*c*). *Note.* Chest decreases in size in initial stages of abdominal inspiration.

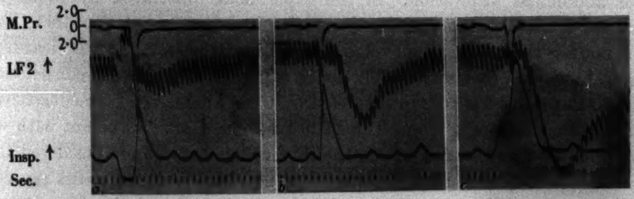

Fig. 7. Effect of variation in the initial position of the chest. Subject S. J. S. Symbols as in Fig. 3. Vital capacity of subject 3·7 l. Sections of a single record to show a group of three inspirations each of 1500 c.c.: (*a*) preceded by expiration of approximately 700 c.c.; (*b*) from the normal expiratory position; (*c*) preceded by inspiration of 1000 c.c. (Length of record omitted between (*a*) and (*b*) = 17 sec. and between (*b*) and (*c*) = 15 sec.)

The initial position of the chest

It was observed frequently during the experiments that if a deep inspiration were taken very slowly, the latent period of the finger response was greatly increased. A similar observation was made by Bolton, Carmichael & Stürup (1936). This phenomenon was further analysed by splitting the inspiration into two stages. The lungs were partially filled with air very slowly, so that no vaso-constriction occurred; then, after a short pause, a further small volume of air was inspired at a rate sufficient to cause constriction of the finger. This latter inspiration was found to be without effect on the finger, when taken from the normal expiratory position.

In one subject, the attempt was made to examine this effect quantitatively. The subject breathed a standard volume of air from a spirometer, first after a deep expiration to air, then from the normal expiratory position, and lastly after partially filling the lungs with air. The effect of one such group of three inspirations is illustrated in Fig. 7; in contrast to the small vaso-constriction obtained with 1500 c.c. taken from the normal expiratory position, no constriction at all resulted when this was preceded by a deep expiration, while a large constriction occurred when it was preceded by the slow inspiration of a litre.

From this it appears that an initially expanded position of the chest and lungs favours the appearance of finger constriction in response to an inspiration of standard size.

DISCUSSION

The original object of these experiments was to establish whether inspiratory vaso-constriction was initiated by the transient fall in arterial pressure which accompanies a deep inspiration. It speedily became obvious that this was not the case.

Bolton, Carmichael & Stürup (1936) failed to obtain finger vaso-constriction after abdominal inspiration. In the experiment illustrated by them, the duration of the inspiratory phase of an abdominal deep breath appears to have been not less than 2 sec.; if its volume were approximately 1 l., it is clear from Fig. 5 above, that this breath could not be expected to cause vaso-constriction. Bolton, Carmichael & Stürup suggested that the absence of vaso-constriction in abdominal breathing inclined them to the view that the afferent path was from the chest wall, and not from the thoracic contents, and considered that the reflex was mediated by the intercostal nerves. The experiments reported here do not justify any assumption of this nature. At fast rates of inspiration, the presence or absence of vaso-constriction appears to be related to the amount of air taken into the lungs; it bears no constant relation to the amount of movement of the chest wall, as recorded by a stethograph at the level of the nipple.

With regard to the effects of the initial position of the chest and lungs, these results show the necessity for inspiring from the normal expiratory position, when comparing the reflex effects of different rates and volumes. Large ventilation rates with abdominal breathing are possible, such as the 83 l./min. described by Mills (1946), but these must involve a substantial reduction in functional residual air; if abdominal inspirations are preceded by forced expiration, in order to achieve greater volume, their reflex effects may not be comparable with those of thoracic breaths, taken from the normal expiratory position.

From the experiments described above, it is not possible to draw any conclusions as to the afferent path involved in inspiratory vaso-constriction in man. Further experiments have been performed upon patients with lesions of the spinal cord at various levels, which provide some information on this problem; these experiments have been carried out jointly, and their results will be reported separately (Gilliatt, Guttmann & Whitteridge, 1947).

SUMMARY

1. Vaso-constriction in the finger after a deep breath has been studied in normal subjects. In some experiments, systolic pressure has also been recorded.

2. Vaso-constriction occurs after a voluntary deep inspiration, or passive inflation of the chest with air under positive pressure. It does not occur after obstructed inspiratory or expiratory efforts, nor after a forced expiration. Respiratory fluctuations in systolic pressure show no constant relation to finger vaso-constriction.

3. From this it is concluded that inspiratory vaso-constriction is not a pressor reflex initiated by the fall in blood pressure which accompanies inspiration.

4. The effect of increased rate of inspiration is to reduce the volume of the breath required to produce vaso-constriction in the finger.

5. Abdominal breathing restricts the volume which can be inspired; under these conditions, vaso-constriction of the finger is only obtained with fast rates of inspiration. Such constriction is comparable with that caused by thoracic inspiration of similar rate and depth.

6. The threshold for the reflex response is influenced by the initial position of the chest and lungs.

7. The site of the afferent endings responsible for this reflex remains obscure.

I wish to express my thanks to Mr D. J. Finney, University Lecturer in the Design and Analysis of Scientific Experiment, to whom I am indebted for the statistical analysis of the data contained in Fig. 5. I also wish to thank Dr D. Whitteridge for a large measure of advice and assistance.

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THE EFFECTS OF PANCREOZYMIN AND OF VAGAL NERVE STIMULATION UPON THE HISTOLOGICAL APPEARANCE OF THE PANCREAS

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It has been shown that stimulation of the vagus nerves in the cat increases the output of enzymes by the pancreas without affecting the rate of secretion of juice (Harper & Vass, 1941). The effect of vagal stimulation is therefore best demonstrated against a background of secretion produced by injections of secretin, which does not itself stimulate enzyme production. Harper & Raper (1943) have shown that extracts of the mucosa of the small intestine contain a material, pancreozymin, which stimulates the production of pancreatic enzymes but is free of secretin activity.

The effect of pancreozymin injections therefore resembles that of vagal stimulation, in that it produces a parallel increase in the output of amylase, lipase and trypsinogen (Greengard, Grossman, Woolley & Ivy, 1944) without, in the cat, causing any increase in the rate of secretion of pancreatic juice. There is, however, the important distinction between pancreozymin and vagal stimulation that the activity of pancreozymin is not abolished by the administration of atropine.

The histological changes that occur in the cat's pancreas on stimulation of the vagus nerve have been studied by Sergeyeva (1938), who showed that prolonged faradic stimulation caused a marked depletion of the zymogen granules in the acinar cells and the appearance of stainable material in the ducts of the glands. These results on cats confirm the earlier experiments on dogs by Babkin, Rubashkin & Savich (1909). Mellanby (1925) found that, after secretin stimulation, the pancreatic cells retained their typical resting structure, but Barrington (1941) considered that the histological appearance of the gland after secretin injections suggested a discharge of zymogen granules. Netik (1937) also found that the effect of prolonged administration of secretin was to diminish the number of granules in the pancreatic cells. In none of these previous observations is there any attempt to correlate the histological changes produced by stimulation with an examination of the output of enzymes in the pancreatic juice.

The purpose of the following experiments was to compare the effects of pancreozymin injections with those of vagal stimulation upon the histological appearance of the pancreas, and to correlate the changes with the output of enzymes by the gland.

METHODS

Experiments were performed on 31 cats. Of these, seven were spinal preparations; the remainder were anaesthetized with chloralose (0·075–0·08 g./kg. body weight, intravenously). With the exception of two animals, which were fed before the experiment, all the animals had been fasting for 18–24 hr. before the experiment, with the object of producing a maximal content of zymogen granules in the pancreas. Small pieces of pancreatic tissue, for histological examination, were removed from the tail of the pancreas some time before, and again immediately after, the period of secretin, vagal or pancreozymin stimulation.

In most of the experiments, pancreatic juice was collected by a cannula inserted into the pancreatic duct. In all these experiments the common bile duct was tied off, and, in addition, the pylorus was in most cases occluded. In ten experiments the splanchnic nerves were cut extraperitoneally.

A continuous flow of pancreatic juice of low enzyme content was maintained at a rate of about 1·0 c.c. in 10 min., by regularly repeated injections of Mellanby's secretin. In the experiments in which the effect of vagal stimulation was being studied, the dorsal vagus trunk was sectioned on the lower part of the oesophagus, and the peripheral end of the nerve stimulated through bipolar electrodes, by faradic shocks from a du Bois-Reymond induction coil (secondary coil at 10 cm., 4 V. in primary circuit) for periods of 4 min. with 2 min. intervals. The nerve was kept moist with saline solution and throughout the period of stimulation the electrodes were from time to time moved caudad to an undamaged portion of nerve.

In experiments on the effect of pancreozymin, intravenous injections of 4·5 mg. of an 'alcohol-soluble' preparation of pancreozymin were given at intervals of 12 min.

The amylase content of successive samples of juice was measured by Wohlgemuth's method, and the minute output of amylase $\left(D\,\frac{J}{T}\right)$ calculated by multiplying the diastatic index by the amount of the sample in c.c. divided by the time of collection of the sample in minutes.

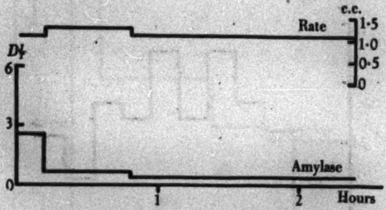
The pieces of pancreatic tissue removed before and after stimulation were fixed in Zenker-Formol solution (mercuric chloride 5 g., potassium bichromate 2·5 g., sodium sulphate 1·0 g., made up to 100 c.c. with distilled water. 5 c.c. of 40% formaldehyde, neutralized with calcium carbonate, were added to the solution immediately before the pieces of tissue were inserted). The tissues were fixed at 37° C. for 15–20 hr., and then post-chromatized in 3% potassium bichromate for 10 days at 37° C. The fixation at 37° C. appears to overcome, to some extent, the difficulty of penetration which has been reported with this fixative (Ramsay, Thomas & Crider, 1943). Of the various stains used, the most satisfactory proved to be Bensley's Neutral Gentian (Bensley, 1911), which stains the enzyme granules a deep purple colour.

In most of the experiments an attempt was made to measure the percentage of the area of the acinar tissue occupied by the zymogen granules. Representative fields of stained sections were projected by means of a microscope on to paper and the clusters of zymogen granules in the cells were outlined. By means of a planimeter the area covered by the zymogen granules was measured and expressed as a percentage of the area of the acini in the field. The edges of the sections, where fixation and staining were usually most satisfactory, were chosen for these measurements. The figures obtained by this method for areas occupied by the zymogen granules give a low estimate for the proportion of the cells occupied by the granules, since they are expressed as percentages of an 'acinar area', which inevitably included the intercellular spaces, although obvious extra-acinar structures, such as islet tissue and blood vessels, were excluded from the calculation. It is also impossible by this method to allow for variations in the concentration of the granules.

RESULTS

There was a good deal of variation from animal to animal in the granule content of the cells in the first piece of pancreatic tissue removed. Moreover, although the animals were fasting, the granule content in many cases was less than had been expected from examination of diagrams in the papers of earlier workers.

Control experiments. In four animals, after the first piece of pancreatic tissue had been removed, the abdomen was closed, and the animals left for 2¾–4 hr. before the removal of the second piece of pancreas. In a second group of four animals the pancreatic duct was cannulated and pancreatic juice collected under secretin stimulation for periods of 3–3½ hr. Pieces of pancreas were removed some time before, and at the end of the period of secretin injections. In these experiments, after the washing out of preformed enzymes in the first sample, the enzyme output fell to a very low level (Text-fig. 1). In the whole group of control animals, examination of the tissue collected at the beginning and at the end of the experiment showed no apparent change in the zymogen content of the pancreatic cells (Pl. 1, A and B).

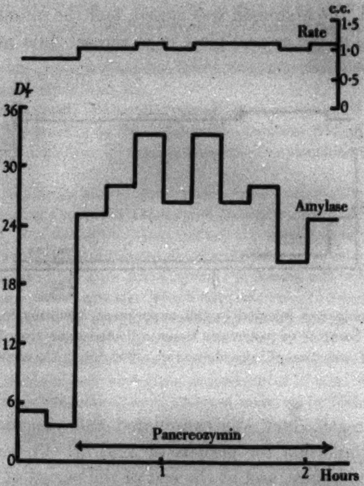


Text-fig. 1. Secretin only was injected in this experiment, resulting in a flow of juice of low enzyme content. Samples of pancreatic tissue collected some time before and immediately after the period of collection of juice showed no alteration in the enzyme granules in the cells (Plate 1 A and B).

Experiments on pancreozymin stimulation. In a series of eight animals the effect of injecting pancreozymin was observed for periods of 1½–3¾ hr. (In most of the experiments, the injections were given for more than 3 hr.) In two of the experiments pancreozymin was given without secretin, and in these no juice was collected; in the remainder, secretin injections were given before and during the pancreozymin stimulation, and the enzyme output in the pancreatic juice estimated. In two experiments atropine sulphate (1 mg./kg. body weight) was injected intravenously before the commencement of the secretin injections.

In one experiment the amount of pancreozymin injected every 12 min. was doubled (9 mg. instead of 4·5 mg.).

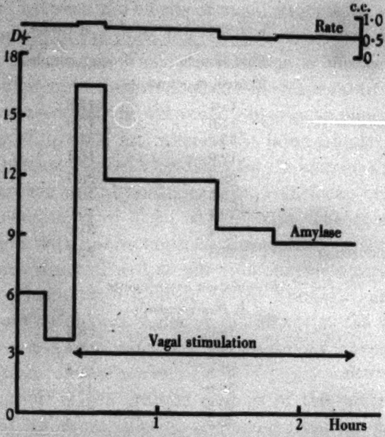
The effect of the pancreozymin injections was to produce a definite increase in the output of amylase, which was maintained throughout the period of stimulation (Text-fig. 2). A comparison of pieces of pancreatic tissue collected before and after pancreozymin stimulation showed a large reduction in the granule content of the pancreatic cells after stimulation (Pl. 1, C and D). Previous injection of atropine did not prevent either the sustained increase in enzyme output, or the reduction in the number of granules.



Text-fig. 2. The flow of pancreatic juice was maintained by injections of secretin. In addition, during the period indicated by the arrows, pancreozymin was given (9 mg. of an 'alcohol-soluble' preparation every 12 min.) resulting in a sustained increase in the output of amylase. A comparison of pieces of pancreatic tissue removed some time before the collection of juice and at the end of the period of stimulation showed a conspicuous diminution in the granule content of the piece collected after pancreozymin stimulation (Plate 1 C and D).

Experiments on vagal stimulation. In nine animals, the effects of vagal stimulation were studied. Stimulation was carried on for periods of 1½–3¾ hr. (in only two animals was the period of stimulation less than 3 hr.). In two animals, no secretin was injected, and no juice was collected; in the remainder, secretin was injected, and the enzyme output estimated. In one experiment the animal was atropinized before the secretin injections and vagal stimulation were started.

Vagal stimulation, like pancreozymin injections, increased the amylase output by the pancreas at the beginning of the stimulation period, but the increased level was not so well maintained as it was with pancreozymin stimulation (Text-fig. 3). The granule content of the pancreatic cells after vagal stimulation was very definitely reduced compared with the control piece of tissue removed before stimulation (Pl. 1, E and F). In contrast to its effect on pancreozymin stimulation, previous injection of atropine completely abolished both the increased output of enzymes and the reduction in enzyme granules on vagal stimulation.



Text-fig. 3. The flow of pancreatic juice was maintained by injections of secretin. During the period indicated by the arrows the dorsal vagus trunk was stimulated in the thorax, resulting in an increase in the output of amylase. Pieces of pancreas were removed some time before the collection of juice and at the end of the period of stimulation. The zymogen granule content was much reduced in the piece collected after vagal stimulation (Plate 1 E and F).

Stainable material was not observed in the ducts of the pancreas after vagal or pancreozymin stimulation, even in those experiments in which no secretin was given, and there was probably little or no flow of liquid which might have washed out the ducts.

In the earlier experiments the decision on whether vagal or pancreozymin stimulation had reduced the granule content of the pancreas depended on subjective impressions of the sections before and after stimulation. As this procedure was open to personal error, the method of estimating the area occupied by the granules by planimeter measurement of drawings of the sections was later substituted. The percentages of the total area of the tissue occupied by zymogen granules before and after stimulation, and the percentage reductions in the post-stimulation compared with the pre-stimulation samples of tissue are given in Table 1. In the control group of animals there is on the average no difference between the samples collected before and after stimulation. In both the vagal and pancreozymin groups the granule content is reduced, on the average, by about half. The statistical significance of the difference between the means of the control group of experiments and the vagal and pancreozymin groups was examined by applying Fisher's *t* test to the results (Fisher, 1934). The difference was found to be easily significant.

Table 1

Experimental procedure	% of section occupied by granules		% increase (+) or reduction (−) in second sample compared with first
	First sample	Second sample	
	Control experiments		
No stimulation	14·4	12·8	−11·1
	11·0	12·6	+14·5
	35·6	37·8	+ 6·2
	19·0	16·1	−15·3
Secretin only	30·2	25·7	−14·9
	15·5	16·9	+ 9·0
	20·4	25·0	+22·5
	28·4	31·6	+11·2
Secretin and vagal stimulation after atropine	25·3	22·1	−12·6
	Stimulation experiments		
	A. Pancreozymin		
Pancreozymin alone	18·9	8·4	−55·5
	13·9	5·4	−61·2
Secretin and pancreozymin	37·8	21·0	−44·4
	36·6	12·7	−65·3
	30·2	25·7	−14·9
	40·5	7·4	−81·7
Secretin and pancreozymin after atropine	35·5	16·4	−53·8
	26·0	5·2	−80·0
	B. Vagal stimulation		
Vagal stimulation only	39·6	12·6	−68·2
	10·8	9·9	− 8·3
Secretin and vagal stimulation	38·4	13·6	−64·6
	42·1	19·0	−54·9
	24·5	4·0	−83·7
	18·6	12·3	−33·8
	60·3	31·9	−47·1
	26·6	23·0	−13·5

Average change in granule content of second sample compared with first sample;

Control experiments: +1·1%.

Stimulation experiments: Pancreozymin, −57·1%; vagal stimulation, −46·8%.

Test for significance of the difference of the averages of the control and stimulation groups of experiments: $t=6{\cdot}00$. (For a group of 25 experiments the difference is easily significant if t is greater than 2·50.)

DISCUSSION

There seems no doubt from these results that sustained vagal or pancreozymin stimulation of the pancreas, in addition to increasing the output of enzymes in the pancreatic juice, diminishes the number of zymogen granules in the pancreatic cells, and that secretin, on the other hand, neither stimulates enzyme output by the gland nor diminishes the granule content of the cells. Barrington's report (Barrington, 1941) that secretin stimulates enzyme production can be explained on the ground that his secretin contained some pancreozymin.

The parallelism between the effects of vagal stimulation and pancreozymin on the enzyme output in the juice and the granule content of the cells is complete, except on two points. First, the hormone is effective after the intravenous injection of atropine, which abolishes the response to vagal stimulation. Secondly, the increased enzyme output in response to pancreozymin is maintained for periods of up to 3¾ hr., whereas the later samples of juice collected under vagal stimulation showed a tendency for the enzyme output to diminish. It is doubtful whether any physiological significance can be attributed to the fall, under the conditions of these experiments. The animal was kept on artificial respiration, with its chest opened, throughout the period of stimulation of the dorsal vagus trunk. The diminution in enzyme output may have been due either to a general deterioration of the animal's condition, of which however there were no obvious signs, or to damage to the vagal trunk itself, although the nerve was kept moist and the electrodes moved down from time to time on to a fresh piece of nerve.

The wide variation in the granule content of the control sample of the pancreas collected before stimulation is obvious from the figures in Table 1. The differences are greater than could be accounted for by variations in the density of packing of the granules. This variation was unexpected, in view of Heidenhain's statement (Heidenhain, 1875) that the pancreatic cells in fasting animals are filled with granules. Although there was no doubt that the granules were markedly reduced after vagal stimulation or pancreozymin, we rarely observed the almost complete disappearance of granules described by previous observers after vagal stimulation. This may be due to the shorter period of stimulation which we employed. Sergeyeva (1938), for example, continued stimulation for as long as 6 hr. compared with a maximum of 3¾ hr. in our experiments.

SUMMARY

1. The effects of secretin, pancreozymin and vagal stimulation upon the zymogen granule content of the cat's pancreas have been studied together with the enzyme output in the juice.

2. Secretin administration brings about the secretion of a juice of low enzyme content, and does not affect the granule content of the cells.

3. Stimulation of the dorsal vagus trunk and the administration of pancreozymin each result in an increased output of enzymes in the juice, and a diminution in the granules in the cells.

4. The increased enzyme output in response to repeated doses of pancreozymin has been maintained for periods up to 3¾ hr.

5. The increased output of enzymes in the juice, and the alterations in the histological appearance of the pancreas in response to pancreozymin are not affected by injection of atropine, which abolishes the effect of vagal stimulation.

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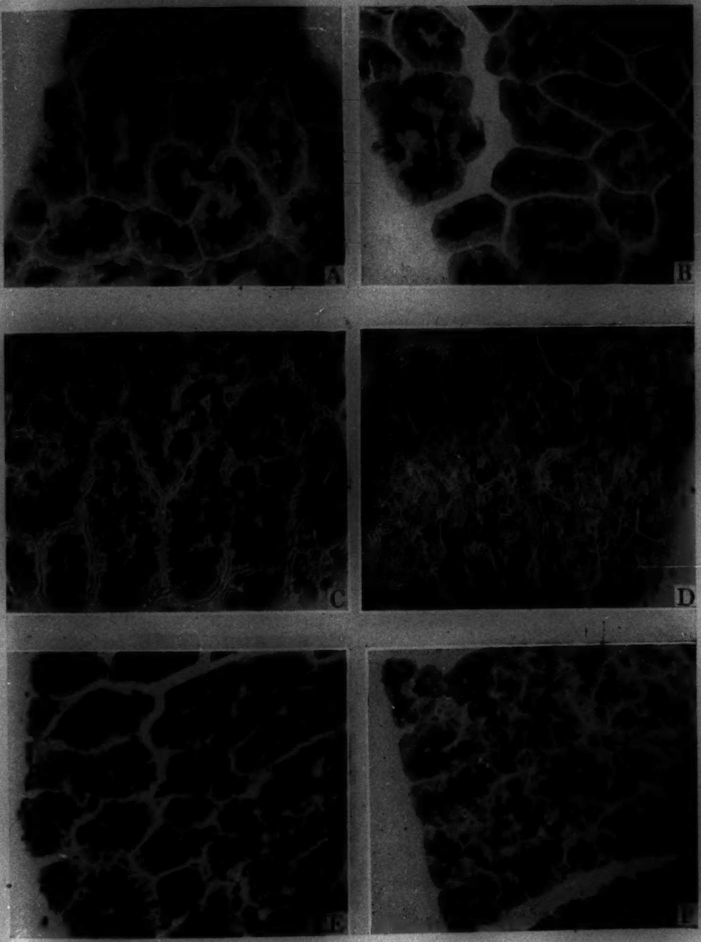
EXPLANATION OF PLATE

A. Pancreatic tissue removed before stimulation by secretin. B. Tissue from the same pancreas after secretin stimulation had produced the secretion of a juice of low enzyme content. There is no alteration in the number of enzyme granules in the cells.

C. Pancreatic tissue collected before stimulation by secretin and pancreozymin. D. Tissue from the same pancreas after stimulation by pancreozymin which resulted in an increase in the output of enzymes in the pancreatic juice. The granules in the cells are clearly less after the stimulation.

E. Pancreatic tissue collected before combined secretin and vagal stimulation. F. Tissue from the same pancreas after vagal stimulation, which increased the enzyme output of the pancreas. After vagal stimulation the granules in the pancreatic cells are much diminished.

(Magnification: A, B, C and D × 500; E and F × 330.)



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SENSITIVITY OF THE SMALL INTESTINE

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In the earliest days of experimental physiology it was shown that the properties of the deep and surface sensory fields were different. William Harvey in 1668 noted that touching the human heart caused no sensation. von Haller (1755) demonstrated that many viscera could be severely injured without evidence of pain, both in man and in other animals.

With regard to the sensitivity of the small intestine, modern work has led to contradictory concepts. Sherrington (1906), in his analysis of the sensory fields, recognized an intero-receptor area including the intestine. This definition was based on the demonstration that stimulation of the viscera produced reflex responses in animals. To Ross (1888), Mackenzie (1920) and Morley (1931) the hollow viscera could be the source of nerve impulses provoking pain sensation, although these authors differed in their views on the physiological basis of the pain. On the other hand, Lennander (1902) again affirmed the apparent insensitivity of the intestine to such trauma as cutting, crushing, burning and stretching. No sensation was provoked by these stimuli applied to intestine exposed at operation, using local anaesthesia. Lennander suggested that pain accompanying disease of a hollow viscus is due to irritation of parietal peritoneum and of the sensory endings of cerebrospinal nerves therein. This would imply that the intestine lacks the neural apparatus which subserves pain sensation. Hurst (1911) attempted to bridge the gap between these concepts by invoking tension as an adequate stimulus for pain endings, but Lewis (1942) pointed out that destructive treatment could scarcely fail to stimulate all nerves and nerve endings in the intestine. In the experiments of Lewis & Kellgren (1939) it was found that pinching the intestine caused no reflex movements of skeletal muscle in spinal cats, whereas pinching the pancreas did so. In both instances there was rise of arterial pressure. Lewis (1942) has suggested that the apparent sensitivity of the intestine to some forms of stimulation and not to others depends upon the spread of the stimulus to adjacent mesentery. This does raise the question whether the intestine is sensitive to the same sorts of stimuli which affect the adjacent mesentery. Meyer (1919) obtained results in lightly etherized cats which suggested that pain arose only from the mesentery and not from the intestine. The pain-provoking stimuli—spasm induced by barium chloride, and distension—caused no response of the animal if the mesentery was protected from stretching. Certainly, whatever may be discovered about intestinal sensitivity, the mesentery is highly sensitive.

In the following paper an account is given of an experimental attack upon the problem of intestinal sensitivity. The principle of the work has been to record reflex actions following intestinal stimulation, taking care that the stimulus was confined to the intestine and that the preparation was responsive to any afferent impulses set up.

METHODS

Certain reflex responses were used as tests for the production of afferent impulses by nerve endings in the intestine. These were (*a*) pupil dilation in chloralosed cats (McDowall, 1925; Bain, Irving & McSwiney, 1935); and (*b*) leg movements and rise of arterial pressure in decerebrated-chordotomized cats (Downman & McSwiney, 1946).

In the first group of animals, chloralose 60–80 mg./kg. body weight, was injected intravenously during preliminary ether anaesthesia. In the second group of animals, the spinal cord was divided between the eighth cervical and first thoracic spinal roots after removing the first thoracic lamina and immediately afterwards the brain above the superior colliculi was removed through a trephine hole in the cranium. Bleeding from the severed cerebral vessels was controlled by tying both common carotid arteries and by temporarily compressing the vertebral arteries. The animals were left for 3 hr. to excrete the ether. Leg movements were observed with the naked eye, and pupil dilatation was observed through a low-power microscope; change of arterial pressure was recorded from a cannula in one carotid artery. The intestine was exposed by an incision through the linea alba, or by an incision through the belly wall in the right flank running forward from the renal area parallel to the lowest ribs. The latter incision exposes duodenum, jejunum, pancreas, and upper part of the colon. When the intestine was drawn out through the incision it was kept warm and moist by applying pads soaked in warmed 0·9% sodium chloride solution, or by immersing the trunk of the animal in a deep bath of Locke's solution (formula in Bain, 1938) at 37–38° C.

RESULTS

Entry of nerve impulses into the spinal cord may be signalled by dilatation of the pupil, rise of arterial pressure and movements of the hind legs. In this work these reflex responses have been used as an index of the production of afferent impulses on stimulating the intestine or mesentery. It may be remarked, in parenthesis, that the absence of such responses does not necessarily signify that no nerve impulses were set up by the applied stimulus. As pointed out in a previous paper (Downman & McSwiney, 1946), the condition of the central nervous system determines whether or not viscero-motor reflexes are obtainable. Deterioration of the preparation is shown by a loss of reflex leg movements upon pinching the intestine, although this stimulus still elicits a rise of blood pressure, showing that afferent nerve fibres have been stimulated. During these experiments the impression was gained that the chloralosed cat might respond to weaker mechanical stimuli than did the acute spinal animal. Otherwise the results obtained in the two preparations were similar.

Stimulation of Small Intestine

Mechanical stimulation. Stimulating any region of the small intestine mechanically sets up afferent impulses in mesenteric nerves, these being signalled by reflex responses. The effective stimuli include compressing or cutting the intestine and also scratching or rubbing its surface with the pointed or blunt ends of a needle. Distension with a balloon is also effective.

The effectiveness of compression was shown in the following ways. The intestine could be pinched with the tips of blunt dissecting forceps or with Halsted's forceps, the tips of the blades being covered with thin rubber tubing to prevent the serrations on the blades breaking the surface. The pinches were effective if the blades of the forceps lay across the intestine or if the blades were directed lengthwise along the gut. Using a pair of Babcock's tissue forceps it was possible to pinch the whole thickness of the gut, over a length of 1 cm., at any site between the free edge of the intestine and the edge bearing the mesentery. It was found that pinches were equally effective when made at the free edge, or near the mesenteric attachment or at any intermediate place. No attempt was made to calibrate the strength of the pinches, but responses followed pinches which could be borne comfortably on the webs of the fingers and which produced no rupture of the serosa, subserosa or muscle. As described in a previous paper, evidence of deterioration of the decerebrated-spinal preparation was the need to pinch the gut more strongly to elicit reflex responses; chloralosed cats tended to show increasing threshold to distension and progressive widening of the pupil. Under these circumstances the threshold pinches had to be so strong that the muscle layers were felt to break beneath the blades of the forceps.

Cutting the intestine was an effective stimulus. This was shown by cutting with a razor-sharp blade along the 'watershed' line at the free edge of the intestine, making an incision which was generally 1–2 cm. long. Cutting lengthwise anywhere between the free and the mesenteric border was effective, but away from the watershed line larger blood vessels were divided and produced copious bleeding. Comparing the reactions of different animals it was noted that all those sensitive to cutting were sensitive to light pinches, but in others cutting was ineffective when light pinches were still effective. The cuts were effective when only the serous coat and the more superficial muscle fibres were severed.

Scratching the surface of the intestine with a needle point, or stroking it with the blunt head of a needle, elicited reflex responses. The pressure of the needle was sufficient to cause a slight depression of the serosa, and when using the point the serosa was torn, as shown by the subsequent line of punctate haemorrhages. When using the blunt head of the needle no macroscopic damage was caused, but it was noted that a single stroke of the needle was insufficient, five or more consecutive strokes being required. It must be emphasized that the two sorts of stimuli just described were effective in cats which seemed to be very sensitive to other stimuli, and were the first type to become inadequate as the preparation deteriorated.

Squeezing the intestine firmly between finger and thumb without tautening the mesentery elicited vigorous reflex response. Even when the condition of the animal was deteriorating, as shown by rising threshold to pinching, squeezing remained an effective stimulus. Indeed, when the animal did not respond to squeezing about an inch of the intestine firmly, it did not respond to any other stimulus applied to the intestine or adjacent mesentery.

Stimulation by heat. Heating the intestine, by applying a hot body to it, set up afferent impulses. This was shown most easily by very gently dabbing the intestine with pledgets of cotton-wool dipped in hot 0·9% sodium chloride solution. A better method was to touch the surface of the intestine with the bend of a thin silver tube, 2 mm. bore, bent sharply into a U-shape, and through which hot water was sucked as quickly as possible. Using the silver tube applicator it was found that all parts of the intestinal surface, between its free and mesenteric edges, was sensitive to heat. The lowest effective temperature was 46° C.

Cold did not produce afferent impulses. No reflex response followed even the application of ice directly to the intestine, or of the tip of a brass rod, 8 mm. diameter, cooled to $-15°$ C. in ice and salt, although the latter process produced a hard white plaque where the tissue froze.

Stimulation by chemicals. Solutions of potassium chloride 1·3%, sodium chloride 10% and hydrochloric acid 1 N. or 0·1 N. were all effective stimuli. The solutions were applied to the surface of the intestine in pledgets of cotton-wool soaked in the solution without allowing any to run on to and wet the mesentery.

Electrical stimulation. Induced currents from an induction coil were effective at a stimulus strength which produced strong contraction of the gut, and was too strong to be borne comfortably on the human tongue.

Stimulation of the mesentery

All the stimuli which have been described as adequate for the intestine elicited reflex actions when applied directly to the mesentery close to the intestine. In addition, rubbing the surface of the mesentery gently with a finger tip or a moist pledget of cotton-wool was also effective, these stimuli being ineffective when applied to the intestine itself.

Origin of the impulses

It has just been pointed out that stimuli which are effective when applied to the intestine are also effective when applied directly to the mesentery nearby. It may be, and indeed has been, postulated that all the reflexogenic impulses arise by indirect stimulation of the mesentery, the stimulus spreading in some way from the intestine. When, however, care was taken that such spread should be prevented, application of the stimuli to the intestine still elicited reflex responses. The conclusion is drawn that the intestine is itself the source of the impulses. The experimental evidence for this, with some consideration how the stimulus might have spread to the mesentery and how this was prevented, is given below.

Elimination of mechanical spread. The act of pinching, cutting or rubbing the surface of the intestine might affect the mesentery if movement of the gut caused the sensitive mesenteric surface to rub on any adjacent surface or if the mesentery was unintentionally tautened. These stimuli were still effective, however, if the intestine was firmly anchored between finger-and-thumb and the stimulus applied to the exposed outer edge of the intestine; or if the loop of the intestine was slung up from two cotton loops threaded through the intestine wall at places 2 in. apart, leaving the mesentery hanging free. Furthermore, with the mesentery and gut floating freely in the bath of Locke solution, pinches were effective before any local contractions of the intestine were seen and without visible movement of the mesentery. Conversely, moving the gut in the bath to produce considerable side-to-side displacement of the mesentery caused reflex response only when the mesentery was pulled taut.

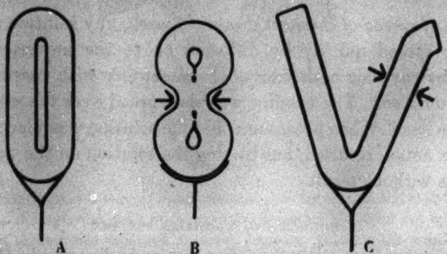

Fig. 1. The small intestine, *A*, when pinched from side to side may bulge and distort the mesenteric attachments, as in *B*. After laying open the intestine along the watershed line, *C*, the wall can be pinched without affecting the mesentery; reflex responses are still obtained. Diagrammatic cross-section.

It may be objected that pinching the intestine caused a slight bulging of the wall alongside the forceps blades. Such bulging might affect the mesentery by causing a separation of its intestinal attachments. If the gut was laid open by incising it along its free edge, one thickness of its wall could be pinched. This still produced reflex responses, but it is difficult to see how the mesenteric attachment could be affected (Fig. 1).

Distending the intestine may fail to be an adequate stimulus while other stimuli applied to the intestine are still effective. In some chloralosed cats it was noted that distending a loop of small intestine with a balloon to 100 mm. Hg pressure elicited no dilation of the pupil. Pinching the same piece of intestine with forceps elicited pupil-dilation. It is difficult to see how the pinch could have affected the mesentery more than did the distension.

Elimination of spread of thermal stimulus. Applying the hot silver tube to the intestine along its free edge could conceivably raise the temperature of the mesentery by direct conduction of heat and by the return of heated blood along the intestinal veins. No attempt was made to counteract this, but it may be pointed out that when the hot tube was placed inside the intestine, temperatures much higher than those which stimulated the outer surface were now ineffective (see later).

Elimination of spread of chemical stimuli. Clearly any solution placed on the intestine may spread quickly by diffusion on to the mesentery. This was counteracted by covering both sides of the mesentery with vaseline which had been warmed until soft. The vaseline was also spread over the mesenteric edge of the intestine itself. When potassium chloride solution was placed on the bare intestine reflex action resulted, but placing the solution on the vaseline-coated mesentery was without result.

Stimulation of intestinal mucosa

Attempts were made to elicit the reflex responses described. It may be said at the outset that drastic stimuli applied to the mucosa were apparently ineffective. The stimuli included heat, cold, chemical substances including strong irritants, pinching and scraping.

Heat was applied by slipping the silver U-tube, 2 cm. long, into the lumen of the intestine, bringing the rubber tubes connected to it out through the small incision made in the intestinal wall. Heating the tube to 60° C. was ineffective; heating it to 80° C. did elicit reflexes but this temperature is so high that the outer layers of the intestine may have been heated.

Solutions were passed through 3 to 6 in. of the lumen of the small intestine by tying into the intestine two wide glass tubes inserted through short incisions in the wall. Perfusing 1·3% potassium chloride, 10% sodium chloride, 70% ethyl alcohol, 5% zinc sulphate, N. hydrochloric acid gave no results. Running in a 2% suspension of mustard in water, and leaving it in, was also ineffective although it caused the intestine to discharge much mucus from its lumen and the mucosa was reddened and haemorrhagic when examined subsequently. On the other hand, placing any of these on the external surface of the gut produced reflex responses.

The mucosa was scraped roughly with a knife edge, after laying the gut open along its free edge, without result. The mucosa was also pinched with forceps. This was done by laying open the intestine and then pulling up the mucosa from the underlying muscle, when it stripped away easily down to the line where large vessels run in through the muscle layers. Although pinching the mucosa gave negative results, pinching the muscle and serosa from which it had been stripped gave positive results (Fig. 2). It may also be noted that, when testing the sensitivity of the intestine to cutting, the reflexes were produced when the knife blade severed only serosa and the most superficial muscle fibres, leaving the mucosa untouched.

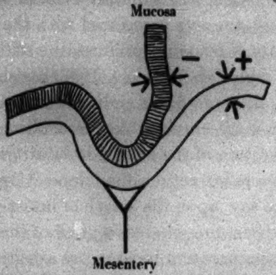

Fig. 2. Diagrammatic cross-section of small intestine, showing mucosa and muscle layers separated by splitting through the submucosa when the intestine had been cut open. Pinching the thickness of the mucosa produced no reflex response; pinching the muscle coats produced reflex responses.

DISCUSSION

These experimental results show that nerve impulses can be set up by applying a wide range of physical and chemical agents to the outer surface of the exposed intestine. The centripetal impulses are able reflexly to activate motoneurones, innervating skeletal and visceral muscle, in the spinal cord and the brain stem as high as the oculomotor nucleus. Further, in the acute spinal animal the occurrence of leg movements and blood pressure changes demonstrates that the centripetal impulses can activate spinal reflex arcs directly without the intervention of higher sensory centres. The role of such impulses in the normal animal is still unknown; indeed, the stimuli employed would not occur in a normal peritoneal cavity, and it is probable that the forms of stimuli used in these experiments are merely non-specific stimuli to nerve endings or nerve fibres. It is possible that these same nerve fibres may be activated in abnormal conditions of the intestine to produce the abdominal muscular rigidity associated with such abnormal conditions. Whether or not these fibres are pain fibres, that is, afferent fibres carrying impulses which ultimately affect high sensory levels to produce a conscious sensation of pain, is a question which awaits an answer.

In animal experiments one must beware of using reflex actions, not involving thalamus or cerebral cortex, to identify pain fibres.

The intestine itself reacts to the stimuli employed, at least when its outer surface is stimulated. Preventing the spread of the stimulating agent to the adjacent mesentery does not abolish the response of the intestine. It is to be concluded therefore that the affected nervous structures lie within the intestine. Broadly speaking the surface of the intestine and the mesentery are sensitive to the same qualities of stimulation, but the latter is also sensitive to the very mild stimulus of gentle rubbing. There is a great physiological difference, however, between the two sensory areas. As pointed out in this and in a previous paper, conduction through the reflex arcs associated with the intestine is lost before conduction through the arcs associated with the mesentery. This is seen experimentally in the loss of responses to intestine stimulation, with retention of those to mesentery stimulation. Sheehan (1932) has shown that the mesentery carries a fine meshwork of nerve fibres ending within the mesentery, and it is possible that these fibres are responsible for eliciting the reflexes from the mesentery when the condition of the animal is deteriorating. The ability of the afferent impulses to cause reflex actions may depend upon the number of nerve fibres involved, that is to say, upon the length of intestine affected. Thus a long scratch was an adequate stimulus whereas a prick of the serosa at one point was inadequate. Also, as the preparation became less sensitive to the stimuli, it was found that responses could be obtained only by stimulating larger areas of the intestine and the effectiveness of squeezing as a stimulus may depend upon the stimulation thereby of a large number of nerve fibres.

Meyer (1919) found that application of barium chloride or distension of the gut by a balloon caused no struggling of lightly etherized animals if care was taken to prevent the spasm or ballooning of the gut stretching the intestinal mesentery. The presence of struggling, when the mesentery was stretched, was interpreted as a reaction which would be accompanied by the sensation of pain in a conscious animal. Lewis & Kellgren (1939) also found that stimulating the intestine in spinal cats did not provoke reflex skeletal muscle movements whereas stimulating the pancreas, with the mesenteric covering, did so. All these authors concluded that there is missing from the intestine one set of nerve fibres—the pain-receiving system. It cannot be proved at the moment that this is not so, but it is very doubtful if one can use an objective reflex movement as a criterion that the same centripetal impulse could provoke a subjective sensation of pain if the pain-perceiving centres were intact and non-narcotized. Furthermore, the absence of responses on stimulating the intestine may represent no more than the deterioration of the reflex arcs associated with the intestine which is provoked by trauma to the intestine.

Confirmation that the intestine is provided with specific sensory fibres, including the small fibres associated with pain end-organs in other parts of the body, comes from electro-physiological investigation of intestinal sensitivity. Both Tower (1933) and Burns (1941) have recorded afferent impulses of low potential from frog gut during strong pressure or other injurious stimuli, and Tower has also recorded such impulses during stimulation of the gut mucosa. More recently Gernandt & Zotterman (1946) have shown that pinching or squeezing cats' intestine provoked slow small spike potentials in the mesenteric nerves, and touching the intestine at a few very localized points on its outer surface produced large rapid spike potentials. These authors, assessing the sensitivity of the intestine in terms of the pattern of nerve impulses, compared the intestine to skin lacking the fast conducting afferents which respond to vibration and light mechanical stimuli as well as to stronger pressure.

The reflexogenic impulses set up in our experiments came from the serosa and muscle layers. Applying destructive stimuli to the mucosa was ineffective, but stimulating the muscle-serosa layer after stripping off the mucosa was still effective. Since scratching was sometimes an adequate stimulus, it is suggested that at least some responsible nervous structure lies within the serosa or subserosa. Whether the impulses were set up by stimulating nerve endings, or whether they were set up by non-specific stimulation of nerve fibres, is an open question.

It should be pointed out that, after completion of this paper, one of the authors (C.B.B.D.) recorded a rise of blood pressure upon pinching the mucosa in chloralosed cats. The muscle coats were stripped back off the mucosa and submucosa without opening the lumen of the intestine. These positive responses re-open the question of mucosal sensibility but do not affect the authors' conclusions concerning the sensibility of the whole intestine or serosa-muscle layers.

SUMMARY

1. In cats, stimulating the exposed small intestine produced dilation of the pupil in chloralosed animals, and leg movements with rise of blood pressure in spinal-decerebrate animals.

2. Effective stimuli were pinching, squeezing, cutting, scratching with a needle point, rubbing with a needle head, heating above 46° C., and applying such solutions as 1·3% potassium chloride, 1 N. or 0·1 N. hydrochloric acid or 10% sodium chloride.

3. Ineffective stimuli were local cooling or freezing, and rubbing the surface gently with the finger tip.

4. These stimuli were effective when the possibility of their spreading on to the adjacent mesentery was prevented.

5. Attempts to elicit reflexes by application of heat or irritant chemicals to the mucosa were ineffective in this series of experiments.

6. It is concluded that the intestine has a sensory nerve supply which is anatomically and physiologically distinct from that of the adjacent mesentery.

7. No conclusion can be drawn as to the nature of the nervous structures stimulated. The relationship between the reflexogenic impulses and pain impulses is discussed.

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THE ACTION OF PHOSGENE ON THE STRETCH RECEPTORS OF THE LUNG

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Since the classical paper of Laquer & Magnus (1921), no attempt seems to have been made to account for the respiratory effects produced during exposure to phosgene. This work of Laquer & Magnus has been used to support one of the current hypotheses on the nature of dyspnoea, that it is due to sensitization of the stretch endings in the lung (Christie, 1938). This hypothesis seems to be contrary to a number of experimental observations (Bülbring & Whitteridge, 1945; Whitteridge & Bülbring, 1944; Walsh & Whitteridge, 1945). On the other hand, the view put forward by Schmidt (1941) that dyspnoea is due to excitation of excito-inspiratory fibres throughout the respiratory cycle, though satisfying, is based on very tenuous evidence of the nature of these fibres. It was therefore thought desirable to observe directly the effect of exposure to phosgene on the activity of pulmonary stretch endings.

METHODS

Single-fibre preparations of pulmonary stretch fibres have been obtained by dissection of the vagal trunk in the neck in cats. In decerebrate animals, the fibres were dissected from a slip of the trunk, the larger part of which was preserved. The nerve impulses were made audible by a conventional amplifier and loud speaker and recorded with a cathode-ray tube and camera. The volume changes in the chest in the spinal animals were produced by ventilation with an all-metal respiratory pump of known stroke volume. Decerebrate cats were enclosed in a metal plethysmograph with the tracheal tube opening externally, and the volume changes in the plethysmograph were recorded by a Krogh spirometer. Its excursions were checked by introducing known quantities of air into a closed circuit made by the spirometer, the plethysmograph and the respiration pump. Owing to the inertia of the moving parts of the spirometer and to wave formation in the water in the tank, amplitudes recorded at rates above 90 per min. are unreliable.

Approximately known concentrations of phosgene vapour were prepared by introducing measured quantities of pure phosgene into partially evacuated dry bottles of about 15 l. capacity, and then allowing air to enter. The animals were made to rebreathe from these bottles. As the exposure was limited to few minutes no attempt was made to absorb carbon dioxide. Control observations were made on the effect of rebreathing from the bottles in the absence of phosgene.

All animals were given 2 mg. atropine subcutaneously. After decapitation or decerebration under ether, time was allowed for the anaesthetic to blow off before beginning observations. When it was necessary to prevent spontaneous movement in decerebrate animals, small doses of nembutal, or preferably chloralose, 10 mg./kg. were given. In some experiments, pleural pressure below the sternum was recorded by a wide-bore needle with air transmission to a sensitive membrane manometer. Activity in the diaphragm was sampled by a hypodermic needle electrode pushed into the xiphisternal head and tied into position.

RESULTS

The effect of phosgene on pulmonary stretch endings was first followed in spinal cats ventilated artificially with constant volumes of air. In different experiments, the phosgene concentration varied from 1/50,000 to 1/2000, with an exposure of 5 min. Preparations which gave the simple response to stretch attributable to a single unit were made from the vagus and on these the effect of phosgene was compared with that of various volatile anaesthetics administered immediately before or after. Although the usual increases in the response of the stretch endings to inflation were seen with the anaesthetics (Whitteridge & Bülbring, 1944), there was no consistent change during exposure to phosgene. The fluctuations in the rate of discharge during exposure were considerably greater than they were in the control period beforehand (Fig. 1). Two of the animals developed pulmonary oedema, and one died within 2 hr. It therefore seemed that the dosage was adequate.

These negative results could perhaps be attributed to the unphysiological condition of the respiratory mechanisms in spinal cats, and it was felt that the observations should be extended to cats which were breathing spontaneously. Decerebrate animals were used with both vagi preserved as far as possible, with a small slip cut from the side of one vagus for recording purposes. When a single-fibre preparation was on the electrodes, the cat was enclosed in the plethysmograph, which was made airtight, with the tracheal tube connected to the external air. The tracheal tube could also be connected through valves to wide-bore tubes ending at opposite ends of the reservoir bottle. A side tube connected to a small balloon minimized respiratory pressure fluctuations. Control observations on the effect of rebreathing from the bottle when it contained room air, showed that there was a decrease or an increase in the respiratory rate of not more than 8 breaths per min. after 5 min., with an increase in the volume of air in the chest at expiration, namely the functional residual air, of 5–10 ml. Of fourteen such experiments, phosgene had no effect on the rate or depth of respiration in six. In the remaining experiments there was always an increase in the rate of respiration and in the functional residual air, but the depth sometimes increased and sometimes decreased. There was always an increase in the minute volume of air breathed. On occasion, the increase in the functional residual air amounted to as much as 70 ml. After removal of the phosgene, the rate and depth returned to their initial values, but the functional residual air remained at a higher level for some time.

Some attempt was made to discover the nature of this insensitivity to phosgene shown by six of these decerebrate cats. It did not seem to be related to the level of the transection, and its appearance was unpredictable. Insensitive animals tested were also found to be insensitive to trichlorethylene and to carbon dioxide, neither of which modified the rate or depth of respiration.

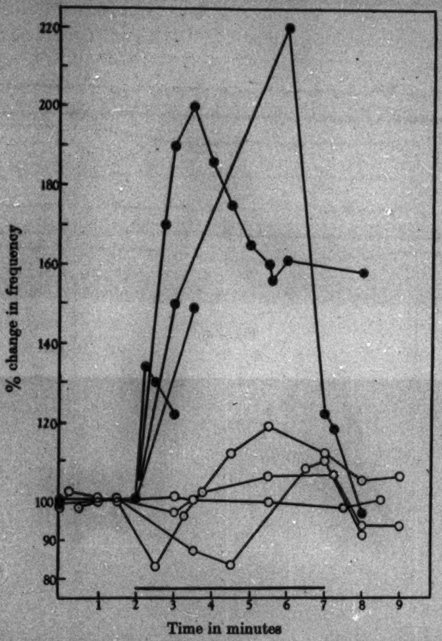

Fig. 1. Spinal cats ventilated with constant volumes of air. Changes in peak frequency of discharge of stretch fibres as percentage of normal, plotted during exposure to phosgene (○), and to cyclopropane, chloroform and ether (●). Phosgene and the anaesthetics were administered for 5 min., except in those cases in which the return of the fibre activity to the base line is not shown.

The decerebrate cats were exposed to concentrations of phosgene of 1/10,000 to 1/5000. Two of those which showed an immediate respiratory acceleration were allowed to survive for 4–6 hr. The resting respiratory rate rose steadily during this time to 40–60 per min. Bilateral vagotomy reduced the resting respiratory rate to 20 per min., a rate which is perhaps within normal limits. After bilateral vagotomy, further exposure to phosgene had no more effect on respiration than had rebreathing from the bottle containing room air. Cooling of both vagi to 0° C. during the administration of phosgene reversibly abolished the respiratory changes, and section of the vagi and the injection of nembutal

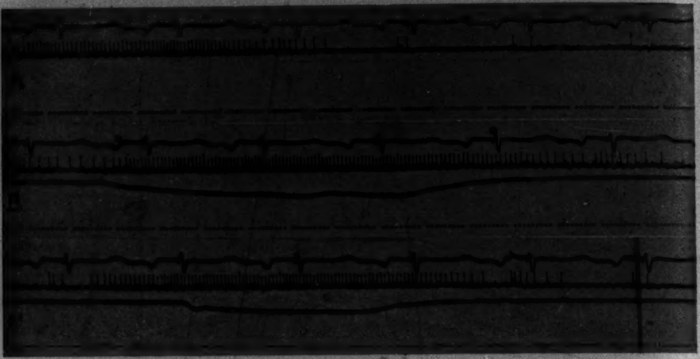

Fig. 2a.

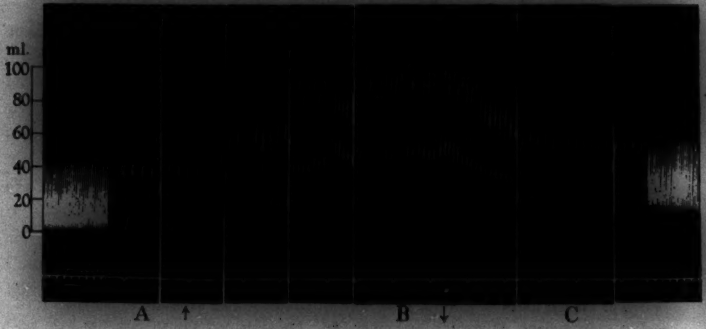

Fig. 2b.

Figs. 2a, 2b. Decerebrate cat, phosgene 1 : 7500 administered for 4 min. from ↑ to ↓. A, B and C in Fig. 2a correspond to A, B and C in Fig. 2b. In Fig. 2a, B and C, from above down, are records of the E.C.G. (inverted), a vagal stretch fibre, the intra-pleural pressure (a fall = more negative pressure), and time in 1/100th and 1/10th sec. The pleural pressure record is omitted in A, Fig. 2a. Fig. 2b is a record of the respiration from the body plethysmograph. Time in sec.

(10 mg./kg.) abolished them irreversibly. It was therefore evident that an intact vagus nerve was essential for the appearance of these phenomena, and an attempt was made to find signs of increased centripetal activity in its fibres.

As the amount of air in the chest was likely to increase considerably during these experiments, it was first necessary to determine the effect on the ending of an increased lung volume without phosgene. This was done by blowing measurable volumes of air into the chest, or by making the animal breathe more deeply by administering mixtures containing increased amounts of CO_2. By itself, CO_2 has no effect on the sensitivity of endings (Adrian, 1933). The peak frequencies of discharge were then plotted against the change in lung volume. An increase in the slope of this curve would then indicate an increase in the sensitivity of the ending, while the vertical displacement of the curve, without change in slope, would indicate a change in the initial level of excitation of the ending.

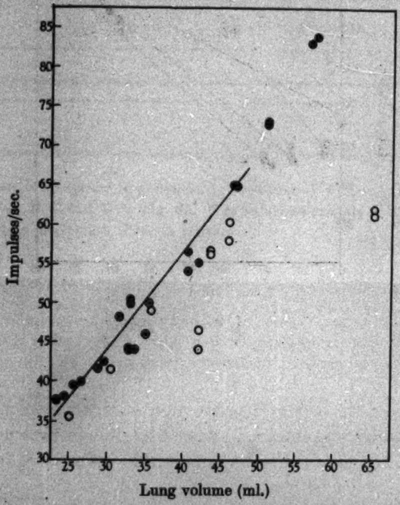

Fig. 3. Decerebrate cat, exposed to 10% O_2 and 10% CO_2 (●), to phosgene 1 : 10,000 (⊙) and to 1 : 5000 (○). Peak frequency of discharge of a stretch fibre plotted against changes in lung volume. In calculation of the regression line for CO_2 the points from the four largest breaths have been omitted.

In general, small increases and small decreases in sensitivity have been seen during and after exposure to phosgene, but there have been no changes comparable with those seen with the volatile anaesthetics (Whitteridge & Bülbring, 1944). In the experiment shown in Fig. 3, for example, with the lower concentration of phosgene, there was no change at all in the activity of the ending at a time when the respiratory acceleration was well marked. With higher concentrations of phosgene, and resulting large increases in the functional residual air, the endings undergo a considerable amount of adaptation. In Fig. 2 the discharge ceases at expiration even at the height of the effect of phosgene, although the lungs then contain as much air as at the peak of inspiration before the administration of phosgene. Adaptation of endings during the maintained increase in functional residual air due to phosgene is also seen in Fig. 4. Here the ending continued to discharge throughout expiration, and its activity decreased, although the lung volume at the end of expiration had increased by 20 ml.

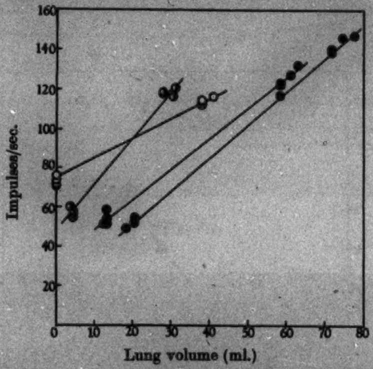

Fig. 4. Decerebrate cat, before (○), during (●), and after (◑), exposure to phosgene, 1 : 7500. Peak frequency of discharge of a single stretch ending plotted against change in lung volume. This fibre continued to discharge throughout expiration.

Some evidence of the mode of production of this conspicuous increase in functional residual air was obtained by recording the intrapleural pressure and the activity of motor units in the diaphragm. The intrapleural pressure usually becomes more negative both at inspiration and at expiration, and the diaphragm goes into continuous activity (Fig. 5). After exposure, the changes in intrapleural pressure and diaphragmatic activity both persist to some extent, as does the increase in the functional residual air. During asphyxia, similar increases in functional residual air have been reported by Verzar (1946) and by Harris (1945).

DISCUSSION

The effects of phosgene on the lung tissue are seldom evenly distributed and, in these experiments, patchy collapse and compensatory overdistension of alveoli was frequently seen in sections of the lungs, even when there was no visible oedema. Some increase in the fluctuations in the rate of discharge of stretch fibres is therefore to be expected, and it is necessary to observe activity in as large a number of fibres as possible. In the spinal cats, there was no evidence of sensitization of stretch endings, and, in the decerebrate animals, respiratory acceleration occurred when there was no change in the activity of the stretch fibre observed. In the other decerebrate cats it is only possible to

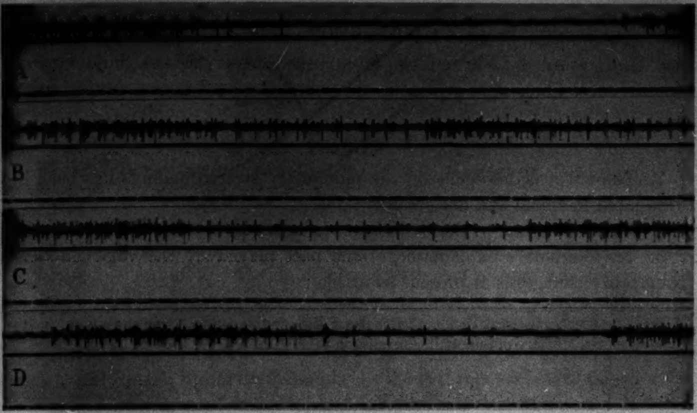

Fig. 5*a*. Decerebrate cat, exposed to phosgene, 1 : 8000 from ↑ to ↓. A, B, C and D in Fig. 5*a* correspond to A, B, C and D in Fig. 5*b*. Fig. 5*a* shows records of diaphragmatic activity beginning with an inspiration. Time in 1/10 sec.

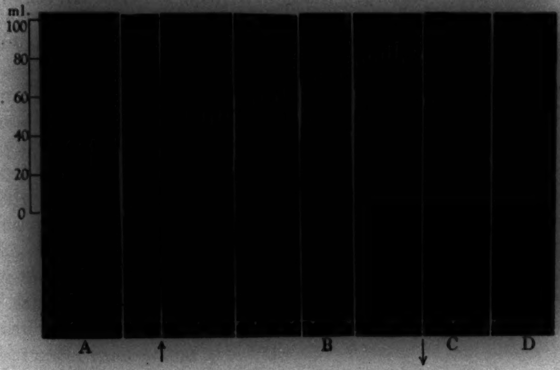

Fig. 5*b*. Records of respiration from the body plethysmograph.

say that if there was any sensitization it occurred at a rate slower than the adaptation of the endings. There is, therefore, no experimental support for the theory of the nature of dyspnoea advanced by Christie (1938). Although a number of other fibres of pulmonary origin exist in the cervical vagus (Partridge, 1939; Hammouda, Samaan & Wilson, 1943), some of whose electrical activity has been recorded (Whitteridge, unpublished observations), unfortunately none of these fibres were present in the vagal slips used in these experiments.

SUMMARY

1. The effect of phosgene on the pulmonary stretch endings has been investigated in spinal and decerebrate cats.

2. No sensitization of these fibres has been seen.

3. No alternative explanation of the part played by the vagus in these respiratory changes is at present available.

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THE ACTION OF ADRENALINE ON MAMMALIAN SKELETAL MUSCLE

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Since the original observations of Gruber (1914) and of Orbeli (1923) the effects of adrenaline and of stimulation of sympathetic nerves upon the contractions of frog's and mammalian muscle have been studied in great detail. Both injection of adrenaline and stimulation of the sympathetic can produce an increase in the peak twitch tension, recorded from muscle excited through its nerve. Bülbring & Burn (1940) found that the best reponse was obtained when adrenaline was injected into perfused fatigued muscles, stimulated through their nerves, and that sometimes the muscle tension was doubled. They found that adrenaline had little effect upon the unfatigued neuromuscular system, and that it produced little or no increase in the tension developed by fully curarized muscle stimulated directly.

Some workers have interpreted the response of the directly stimulated muscle to adrenaline as an indication that the sole action of adrenaline is upon the muscle fibres themselves (Michol, 1930). More recently, however (Bülbring & Burn, 1940; Burn, 1945), stress has been laid upon the disparity between the responses to adrenaline of muscle excited through its nerve and stimulated directly. Adrenaline has also been found to augment the tension increase produced by the action of prostigmine and eserine upon muscle stimulated indirectly. In view of these and other facts, recent workers have tended toward the view that the main site of action of adrenaline is the neuro-muscular junction, where it has been supposed to facilitate transmission from nerve endings to muscle (Bülbring & Burn, 1942; Bülbring, 1946).

The experiments described in this paper are mainly concerned with an examination of the electrical phenomena in muscle and were designed to provide further information about the site of action of adrenaline upon the mammalian neuro-muscular system. Our results have given us reason to believe that the action of adrenaline in the fatigued nerve-muscle preparation is primarily upon the muscle fibre itself, and that effects upon neuro-muscular transmission play only a small part, if any at all, in the enhancement of twitch tension.

METHODS

In the few experiments in which cats were used, they were decerebrated under ether anaesthesia and the tibialis anterior was prepared for recording (Brown, 1938).

Most of the experiments upon which this account is based were made on the isolated phrenic-diaphragm preparation of the rat (Bülbring, 1946), and the original technique was followed closely, except that strips of muscle only 2–3 mm. in breadth were usually employed. Electrical recording necessitated some minor modifications which will be referred to later.

The electrical stimuli applied to nerve and muscle were taken from the secondaries of air-cored transformers through the primary of which a condenser was discharged; the discharge of the condenser was controlled by a soft valve. When two or more stimuli were given the interval between them was controlled either electrically or with a Lucas pendulum.

The precise form of the electrodes used for stimulation varied with the needs of the experiments, but, in all instances, the contact with the tissue was through silver wire. In the experiments on cats, the stimuli were applied through bare silver wires, 1 cm. apart, on either side of the sciatic nerve which was tied proximally. The test shocks, given during the course of a tetanus, were applied through a second pair of electrodes placed 1 cm. distal to those used for the tetanus.

The phrenic nerve was usually excited through two of a number of silver wires 1 mm. apart upon which it rested, just above the surface of the fluid bathing the muscle. In the course of the experiments we found that satisfactory stimulation of the nerve or muscle could be effected beneath the surface of the bath by applying a capillary glass tube which was connected to a suction pump and contained in its tip a fine silver wire.

Records of muscle tension were taken with a flat spring, cantilever myograph in the experiments on cats and with a very light torsion myograph in the experiments on the rat's diaphragm. The small tensions developed by the diaphragm and the rapidity of its contraction made accurate recording of the form of the single twitch a matter of some difficulty.

All tension records were made under isometric conditions, the magnification of the movement being × 200 and the biggest final deflexion some 3 cm. The applied initial tension was approximately half the twitch tension.

The action potentials of the muscle were led into a condenser coupled, push-pull amplifier, the resistance between each input lead and earth being 0·5 MΩ. In all experiments, except those in which single muscle fibres were recorded, the time-constant of the circuit was 75 msec. In the experiments on the cat's tibialis anterior, the records were taken with the cat earthed and belly-tendon leads.

The method of recording from the diaphragm varied with the type of experiment. When the gross action potential of the muscle was required, one amplifier lead was connected to the tendon through a silver wire, and the other to the earthed fluid in the bath. The muscle fibres then projected 2–3 mm. above the surface of the fluid in the bath and were kept moist by spray from the vigorous oxygen supply. This method of recording has the disadvantage that the electrical record shows random fluctuations in amplitude which are due to the formation of bubbles on the surface of the liquid close to the muscle, an irregularity which is not remedied, but is even increased by covering the fluid with a layer of liquid paraffin. Another disadvantage of this method is that the electrical record is taken from only a small fraction of the fibres contributing to the total tension. Yet a further difficulty introduced by recording in this way is that the effective distance between the recording electrodes, and consequently the shape of the action potentials, vary with the initial tension to which the muscle is subjected.

When records were taken from very small fractions of muscle, electrodes were applied to the surface of the muscle which was suspended horizontally. An insulated silver wire, with its end cleaned by a transverse cut, was found to give satisfactory records of the electrical activity of a few motor units.

In order to obtain records from single muscle fibres, pore electrodes were used for stimulating and recording. They were constructed by melting and pulling out a piece of 'Pyrex' tubing containing a length of silver wire. These make a satisfactory joint and enable the end of the glass tubing to be ground flat until the diameter of the silver core is of the required size. We used electrodes 0·5 mm. in diameter with a silver core of $5\ \mu$ cross-section. They were held in micromanipulators.

In the figures, no absolute values of either the tension developed by the diaphragm or of the recorded action potential are given. The reason for this is that we used strips cut to suit the needs of each particular experiment; the strips varied in breadth from 1 mm. to 1 cm., although, as stated above, 2–3 mm. was the usual width. The tensions recorded were, therefore, widely different from experiment to experiment. For the same reason, the absolute values of the recorded potentials were without significance; not only did the strips vary in size, but the degree of short circuiting by the bath fluid varied from experiment to experiment.

RESULTS

Experiments on the tibialis anterior of the cat

Preliminary experiments showed that the effect of adrenaline upon the tension developed by a fatigued muscle could be demonstrated easily. Maximal stimulation of the sciatic nerve at frequencies of the order of 30/sec. produces a contraction of tibialis, the tension of which falls steeply to about half its initial value in some 3 min., and then shows little decline for 10 min. or more. There is a corresponding change in the peak voltage of the action potential. In testing the effect of adrenaline we generally interrupted the tetanic stimulation for a period of about 2 sec. and interpolated a single maximal stimulus to the nerve, at a time when the muscle was fully relaxed after the end of the tetanic stimulation. This enabled us to maintain a steady level of 'fatigue' and yet to record the uncomplicated response of the muscle to a single nerve volley.

The intravenous injection of $50\ \mu g.$ of adrenaline during the steady period caused prompt increase of tetanic tension and of the tension produced by the test shock. An increase in twitch tension as great as 40 % may not be accompanied by any change in peak action potential, and we have observed increases in tetanic tension up to 80 %, again without alteration of peak action potential. We have, on occasion, observed small increases in peak action potential running parallel with the increase of tetanic tension, but these were, in any event, irregular and were not observed in the responses to the single volleys. Their significance is discussed later.

These experiments showed that, whatever action adrenaline might have upon fatigued muscle in the intact animal, it was improbable that the recruitment of additional muscle fibres or units played a significant part in the increase of tension. It was, of course, possible that additional fibres were in fact contracting, but that the action potential of all fibres was reduced in such a way as to leave the total potential unaltered. Previous experience of the effects of potassium chloride (cf. Brown & von Euler, 1938) and of quinine upon muscle (Harvey, 1939) has shown that increases in twitch tension may be accompanied by an actual diminution in peak action potential. It was, therefore, obvious that the phenomenon required much more detailed analysis than could conveniently be made on a large muscle with natural circulation, and we consequently continued the investigation on an isolated mammalian nerve-muscle preparation.

Experiments on the isolated diaphragm

The isolated phrenic-diaphragm preparation is more susceptible to fatigue than one with natural circulation; nevertheless, the threshold of its nerve, its nerve action potential, and, indeed, the action potential of the muscle show little change in the course of a day's experiment if stimulation is not maintained for long periods. We have found that stimulation of the nerve with single maximal shocks at a frequency of 2/sec. for some minutes is sufficient for the demonstration of the action of adrenaline. The immediate consequence of repeated stimulation of the nerve with supramaximal shocks at frequencies of 2/sec. is a steady decline in peak tension, and a parallel decline in peak action potential; at the same time, however, the action potential lasts longer. In other words, the action potential, recorded monophasically, becomes smaller in height and broader across the base.

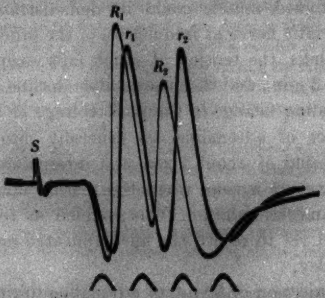

Fig. 1. Rat diaphragm. Action potential recorded from two points of surface, two records being superimposed. *s*, stimulus artefacts. R_1, R_2, responses of fresh preparation to single maximal nerve volley; r_1, r_2, responses of same muscle after 12 min. maximal stimulation of nerve at 2/sec. Time, 1000 cyc./sec. Photograph retouched.

The cause of this broadening appears to be an increase in the temporal scatter of the responses of the constituent fibres of the muscle. We were able to demonstrate this by applying two pore electrodes to points approximately 6 mm. apart on the surface of the diaphragm and feeding the combined action potentials into the same amplifier channel. By placing the electrodes so that one lay near the anatomical entry of the nerve and the other some distance from it we were able to obtain two discrete spikes with very different latencies. Fatigue of the preparation caused a clear increase in the temporal dispersion of the responses (Fig. 1).

Effect of adrenaline on the response to single maximal nerve volleys. The effect of the addition of adrenaline to the bath in doses of 1–2 μg./c.c. Tyrode's solution is an increase in the peak twitch tension, without any consistent change in the peak action potential. There is, on the other hand, a further broadening of the base of the action potential which runs *pari passu* with the change in tension.

The results of a typical experiment are shown graphically in Fig. 2 and examples of the changes in action potential and tension are given in Fig. 3.

It will be observed that the tension records in Fig. 3 suggest that the broadening of the action potential is associated not only with an increase in peak twitch tension, but also with an increased duration of the twitch. Tension records taken at higher speeds have, on occasion, revealed an unmistakable slowing of the tension wave, but this has always been small, and we have not been able to demonstrate it regularly with the methods of recording available.

Effect of adrenaline on the response to two nerve volleys. In these experiments, the nerve was excited with two supra-maximal shocks at various intervals, and the mechanical and electrical responses were recorded. Continued stimulation of the nerve with single and paired stimuli, repeated at 1–2 sec. intervals, had little effect upon the absolute refractory period of the system or upon the recovery curve of the second response. The absolute refractory period of the preparation stimulated through a single electrode 1 cm. from the muscle was about 0·75 msec. at 37° C., and recovery of the second response was complete in 5 msec.

The administration of adrenaline in doses sufficient to cause a clear increase in the twitch tension and in the duration of the first action potential was without effect upon either the absolute refractory period or the recovery curve. Although the peak tension resulting from a pair of stimuli, separated by a small time interval (2–10 msec.), was increased by adrenaline, the contribution of the second response of the muscle to the total tension was unaltered. If, for example, the tension developed by the fatigued muscle in response to a single stimulus was 1 g. and after adrenaline was 1·25 g. we found that the tension for a pair of stimuli 4 msec. apart was increased by adrenaline from 2·5 to 2·75 g. (cf. Fig. 2).

It was clear, in view of the constant broadening of the action potential produced by adrenaline, that more information was needed about the effect of adrenaline on the directly excited muscle and upon smaller contractile units than we had been using.

Effect of adrenaline on muscle excited directly. Preparations of the diaphragm were suspended in the bath and curarine chloride (King, 1935) was added until neuro-muscular transmission was blocked. In the course of the experiment, the completeness of the block was checked by nerve stimulation. The muscle was excited by stimuli from an insulated silver wire applied to its surface

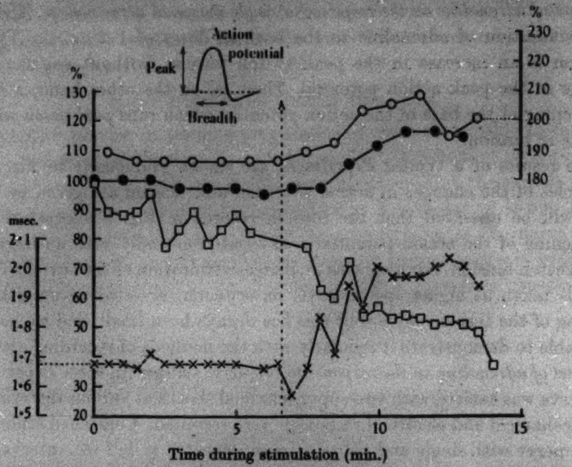

Fig. 2. Rat diaphragm. Responses to maximal nerve volleys at 30/min. ○—○ tension response to two volleys at 2 msec. interval as percentage single response at start. ●—● tension response to single volley as percentage at start. □—□ peak action potential as percentage at start. ×—× breadth of action potential in msec. At arrow adrenaline to give a concentration of 1 μg./c.c.

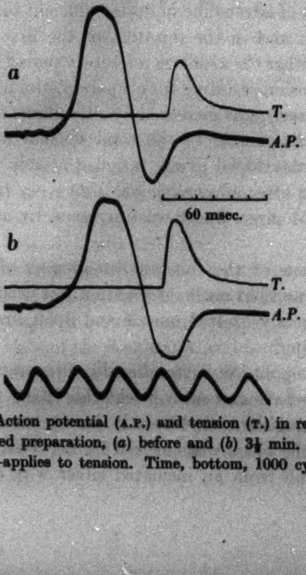

Fig. 3. Rat diaphragm. Action potential (A.P.) and tension (T.) in response to single maximal nerve volleys. Fatigued preparation, (a) before and (b) 3½ min. after adrenaline 2 μg./c.c. Time, top, 10 msec.—applies to tension. Time, bottom, 1000 cyc./sec.—applies to action potential.

beneath the liquid of the bath, and electrical and mechanical records were taken. The stimuli were made as strong as the apparatus could supply, but it must be emphasized that they could not be in any sense maximal. The reason for this is that the fibres of the diaphragm are not more than 5 mm. in length, and any stimulus applied to one group excites that and not adjacent fibres.

Sufficiently reliable records were obtained, however, to make it clear that adrenaline produces, in directly excited muscle, a broadening of the action potential as great as that in muscle excited through the nerve (Fig. 4). The broadening is associated with an increase in the time from stimulus to peak potential.

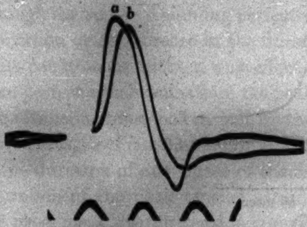

Fig. 4.

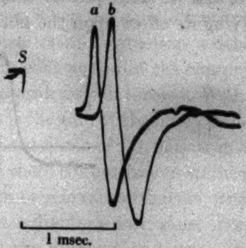

Fig. 5.

Fig. 4. Rat diaphragm. Action potential of fully curarized preparation, stimulated directly. Superimposed records (*a*) before and (*b*) 1½ min. after adrenaline 2 μg./c.c. Time 1000 cyc./sec. The stimulus artefact has been removed.

Fig. 5. Rat diaphragm. Action potential of single muscle fibre, fully curarized, stimulated directly. Superimposed records (*a*) before and (*b*) after adrenaline 2 μg./c.c. The stimulus artefact beginning at *S* has been omitted. Amplifier time constant 7·5 msec. Time 1 msec.

Experiments with single muscle fibres. The experiments on directly excited muscle provided evidence that adrenaline altered the spread of the excitation wave in the individual muscle fibre, and only experiments with single muscle fibres could give direct proof of this hypothesis. The method of stimulation and recording has already been described, but attention should be drawn to the method adopted to ensure excitation and recording of the same single fibre. The electrodes were alined by adjusting them so that the smallest effective stimulus gave the greatest response. We have assumed that when a continuous variation of stimulus strength gave an all-or-nothing electrical response, the response was that of one muscle fibre only. It is easy to avoid exciting the fibre through its nerve terminal, since, when this occurs, the electrical records show the 'immediate' response of the single fibre in question, followed 1 or 2 msec. later by the response of the remainder of the fibres in the motor unit. We have checked a number of our preparations by giving a dose of curarine, sufficient to produce complete curarization, but have invariably found that this left the records of action potential unaltered.

The action potentials recorded were potential differences between that part of the fibre beneath the silver electrode and the earthed fluid in which the whole diaphragm was bathed. Since the end of the silver electrode was insulated from the surrounding fluid by the glass sheath in which it was fused, the action potentials recorded were triphasic. Whatever interpretation is given to the triphasic record, it shows clearly that adrenaline delays the spread of the excitation wave (Fig. 5).

The effect of temperature. The foregoing experiments made it clear that the major effect of adrenaline was upon the speed of conduction of the excitation wave, and we consequently sought another means of slowing the wave and of studying its effect upon the tension.

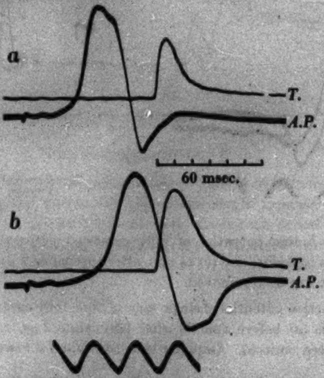

Fig. 6. Rat diaphragm. Action potential (A.P.) and tension (T.) in response to single maximal nerve volleys. Fatigued preparation, (a) at 37·8° C. and (b) at 31·5° C. Time, top, 10 msec.—applies to tension; bottom, 1000 cyc./sec.—applies to action potential.

We found that cooling the muscle produced a slowing of the electrical excitation wave and a corresponding increase in duration and tension of the mechanical response. Fig. 6 should be compared with Fig. 3, when the general similarity of the effects becomes obvious. It will be noted that they differ in one important respect: the latent period of the electrical response is unaffected by adrenaline, whereas in the 'cold' record it is increased in duration.

The experiments which we have described were designed to reveal the site of action of adrenaline upon the neuro-muscular system. Their results gave no evidence that adrenaline affects neuro-muscular transmission, but did point to a direct action upon the muscle fibres themselves. If we assumed this to be the only action of adrenaline, we still were without an explanation of those particular circumstances in which adrenaline increases twitch tension. The experiments described below were designed to test whether the reported actions of adrenaline could be explained in terms of direct action upon the muscle fibres alone.

Artificial desynchronization of the fibre responses in the rat's diaphragm

We sought some feature common to fresh muscle excited through its nerve and to muscle excited directly, because these were the outstanding circumstances in which adrenaline was reported to have little effect upon twitch tension. The degree of synchronization between the contracting fibres appeared a promising line of approach, since we already had evidence that fatigue caused a considerable increase in asynchrony of the fibre responses, when the muscle was excited through its nerve. Assuming perfect synchronization of contraction, a delay in relaxation or an increase in the duration of the contraction of the component units could occur without any effect upon the peak twitch tension. With less than perfect synchronization, the peak tension is the sum of the many different instantaneous tensions reached by the component fibres of the muscle in their various stages of contraction or relaxation. In these circumstances an increase in the duration of any phase of the contraction or relaxation process, without alteration of the peak tension attained by an individual unit, must increase the gross peak twitch tension, provided that the duration of tension in the individual unit is of the same order as the degree of desynchronization between units. Desynchronization can, therefore, only account for the observed facts if the peak tension in the single muscle fibre lasts no longer than 2 or 3 msec., since the observed desynchronization, with the degrees of fatigue we have used, has never exceeded 1 msec. In our experiments adrenaline has never increased the observed duration of the action potential by more than 0·5 msec., and the time to the peak of the twitch has never been observed to increase beyond 2 msec., and only in a few experiments was it demonstrable at all.

It seemed possible, nevertheless, that an artificial desynchronization produced in a fresh muscle might reveal an augmentation of peak tension by adrenaline which was not present when the nerve was excited maximally in the usual way. It also suggested an explanation of the puzzling phenomenon of adrenaline affecting the response to submaximal, but not to maximal nerve volleys in the unfatigued muscle (Bülbring, 1946); submaximal might well be expected to initiate a less synchronous volley than maximal stimuli (cf. Blair & Erlanger, 1935). Bülbring's observations were made by testing the effect of adrenaline first on a series of submaximal twitches, and then on a series of maximal twitches; she found that adrenaline increased the tension of the submaximal twitches but not that of the maximal. We excited the nerve alternately with submaximal square waves, 2·13 msec. in duration, and by maximal induction shocks from our usual stimulator. The long duration of the submaximal stimuli was chosen to imitate those used in Bülbring's experiments.

The effect of adrenaline upon tension was found to be identical in both responses, whether its access to the nerve was excluded or not: a surprising finding in view of Bülbring & Whitteridge's (1941) observation of the effect of adrenaline on the excitability of nerves in the whole animal.

A second method which we adopted was to prepare a diaphragm and cut half way across the nerve trunk some 2 cm. from the muscle. Stimulating electrodes were applied on either side of the cut and close to it. The nerve was excited alternately with a maximal single shock through the electrode nearer the muscle and with maximal paired shocks separated by 0·5 msec. through both electrodes, the point further from the muscle being excited first. With this method, the greatest separation between the volleys is limited by the refractory period of the nerve, usually about 0·75 msec. In order to obtain a greater separation, attempts to split the nerve longitudinally having failed, we adopted the device of making two preparations and arranging them to pull in series, the tendon of one being attached to the costal margin of the one above it. The muscles could then be excited either independently, simultaneously or at any chosen interval.

Artificial desynchronization by all these expedients failed to increase the effectiveness of adrenaline. Our failure to reproduce this effect of fatigue does not necessarily exclude desynchronization as at least one factor contributing to the enhancement of the effect of adrenaline, since the desynchronization which we have observed is almost certainly between the fibres of a motor unit, and that which we produced artificially was of necessity between many motor units. But it seemed improbable that the effect of desynchronization between the responses of fibres in one motor unit could not in part be demonstrated by a desynchronization imposed between one motor unit and the next, many of whose muscle fibres must be intimately mixed.

Action of adrenaline upon the rat's diaphragm stimulated alternately through its nerve and directly

These findings made it seem probable that, despite earlier accounts, contractions of the rat's diaphragm produced by excitation of the nerve and by direct stimulation of the muscle would be equally affected by adrenaline. In order to make a satisfactory comparison between the responses from these two different methods of stimulation it was essential that the direct stimulus should be maximal when the muscle was submerged in Tyrode's solution. Maximal tension responses to direct stimulation were obtained by enclosing a strip of muscle in a short length of 3–5 mm. bore glass tube, open at both ends. The glass tube contained annular silver electrodes at opposite ends of the muscle strip for direct stimulation, and the nerve was led out to the electrodes for indirect stimulation through a small hole in the glass tube. A square wave of about 100 V. and 1 msec. duration applied to the ring electrodes was found adequate for direct stimulation. Stimuli lasting more than 1·5 msec. are liable to produce double responses in the fresh muscle. In all instances the adequacy of the direct stimulus was tested at the end of the experiment with complete curarization.

We found that when a freshly excised muscle is fatigued by maximal stimuli to the nerve at 1–2/sec., there is a parallel decline in the twitch tension resulting from excitation of the nerve and in the tension produced by direct stimulation of the muscle. Adrenaline in doses of 1–2 μg./c.c. bath fluid affects both responses equally. The results of a typical experiment are plotted in Fig. 7. It is of considerable interest that these experiments provided no evidence of neuromuscular block.

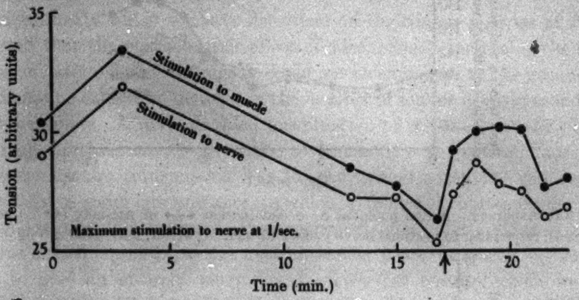

Fig. 7. Rat diaphragm. Tension response to ●—● maximal direct stimulation of muscle and to ○—○ maximal nerve volleys, sampled during continuous stimulation of the nerve at 1/sec. At arrow, adrenaline 2·5 μg./c.c.

The action of adrenaline on partially curarized muscle

Although the preceding experiments showed that adrenaline could produce considerable increases in the twitch tensions of muscles in which no failure of neuro-muscular transmission was demonstrable, it still seemed possible that adrenaline might facilitate transmission when this failed. With this point in mind we examined the action of adrenaline upon the partially curarized isolated rat's diaphragm excited through its nerve, its action potential and tension being recorded simultaneously. Fig. 8 shows the results of an experiment in which a reasonably steady level of partial curarization was attained. The effect upon twitch tension is quite dramatic, but it is accompanied by an increase in peak action potential which is less in degree and duration. We also adopted the more direct technique of recording the tension response of the partially curarized muscle, excited with stimuli applied alternately directly and to the nerve. The results of a typical experiment are shown in Fig. 9. The responses to the direct stimulation show a steady decline as the muscle fatigues, and those to the nerve volleys a similar decline, the origin of which may be muscular fatigue or a change in the degree of curarization. The administration of adrenaline causes an increase in tension, the absolute amount of which is equal in both curves.

Neither of these experiments provided completely convincing evidence that adrenaline is without any effect upon neuromuscular transmission depressed with curarine. In the first, there is a small increase in the peak action potential during the early part of the increase in tension. In our opinion this transient increase of peak action potential does not necessarily indicate that there has been an increase in the number of muscle fibres contributing to the total action potential; like the peak tension, the peak action potential of the whole muscle can be increased by a prolongation, after adrenaline, of the action potential of the single component muscle fibres, if these are firing asynchronously. In the second experiment, there is a change in the ratio of the tension developed from the direct stimulus to that from the nerve volley, but the degree of

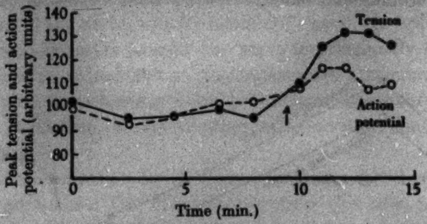

Fig. 8. Rat diaphragm. Action potential o—o and tension •—• of partially (40 %) curarized muscle, responding to maximal nerve volleys 12/min. At arrow, adrenaline 2·5 μg./c.c.

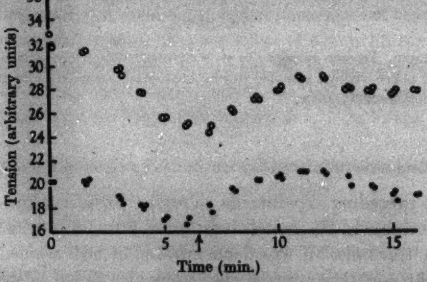

Fig. 9. Rat diaphragm. Tension responses of partially curarized muscle to maximal direct stimulation (o) and to maximal nerve volleys (•). At arrow, adrenaline 2 μg./c.c. Sampled during continuous stimulation at 12/min.

decurarization in terms of relief of neuromuscular block is evidently very small in extent and duration in comparison with the total increase in tension.

The success of experiments of this nature depends upon the maintenance of a steady level of curarization over a considerable period of time. We only partially achieved this, and slow changes in the depth of curarization were taking place in both the experiments we illustrate. We have, in fact, found the maintenance of a steady depression with curarine to be a matter of great difficulty with this preparation, largely because of the slowness of action of the drug and the dependence of its effect upon the precise frequency of the stimuli used either for testing or for fatiguing the tissue.

DISCUSSION

The experiments which we have described on the tibialis anterior of the cat suggested that the characteristic effect of adrenaline in increasing the twitch tension of fatigued skeletal muscle could not be explained on the ground that the adrenaline caused an increase in the number of muscle fibres responding to the nerve volley. A more detailed examination of the effect on the phrenic-diaphragm preparation not only failed to demonstrate any action of adrenaline on neuro-muscular transmission, but has shown that adrenaline can augment twitch tension under circumstances in which no failure of neuromuscular transmission could be demonstrated. The experiments in which the effect of adrenaline was tested upon a partial neuromuscular block produced by curarine failed to give an entirely unequivocal answer for reasons which we have considered above. They made it clear, however, that whatever the effect of adrenaline upon neuromuscular block, it is small compared with its direct action upon the muscle fibre.

Of the precise mechanism whereby adrenaline exerts its action on muscle we have no knowledge. We are, in general, in agreement with the statement made by previous workers that some degree of fatigue is necessary before adrenaline can act, but there were certain irregularities in the behaviour of our preparations which make us uncertain that fatigue is the whole answer to the problem. Adrenaline is known to cause a mobilization of K^+ from animal tissues (cf. D'Silva, 1937; Hebb & Nimmo Smith, 1946), and K^+ has striking effects on both muscle tension and action potential (Brown & von Euler, 1938). The recent observation by Goffart & Brown (1947) that the effectiveness of adrenaline on the isolated diaphragm preparation is a function of K^+ content of the bath fluid suggests that adrenaline may act by causing changes in the distribution of K^+ in the muscle.

The interpretation which we have given to our results differs from the earlier conclusions of Bülbring & Burn (1940). It is difficult, however, to relate our experiments, mainly on isolated tissues, to theirs on intact and perfused muscles excited with brief, tetanic and probably submaximal nerve volleys.

Many indeed of the discrepancies between our findings and those of Bülbring & Burn could be explained by assuming that, in their experiments, adrenaline had increased the plasma [K⁺] and thereby evoked changes in the threshold of excitable tissues.

SUMMARY

1. In order to determine the site of action of adrenaline upon skeletal muscle, its effects have been examined on the tibialis anterior of the decerebrate cat and on the phrenic-nerve-diaphragm preparation of the rat.

2. Although adrenaline causes an increase in the tension response to maximal motor nerve volleys of both preparations, the peak action potential is not increased.

3. Adrenaline causes an increase in the duration of the action potential which runs parallel with the increase in tension.

4. The increase in duration of the gross action potential is due to a retardation of the spread of excitation wave along the individual muscle fibres.

5. No evidence has been obtained that adrenaline owes its effect to the relief of neuromuscular block.

6. The effects of adrenaline on the tension response of an excited muscle appear to be due to a direct action upon the tension developed in the individual fibre.

7. Fatigue increases the temporal dispersion in the response of a muscle to motor nerve volleys, but this cannot be shown to account for the greater effect of adrenaline in fatigued muscle.

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THE EFFECT OF VARIATIONS IN OSMOTIC PRESSURE AND ELECTROLYTE CONCENTRATION ON THE MOTILITY OF RABBIT SPERMATOZOA AT DIFFERENT HYDROGEN-ION CONCENTRATIONS

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In a previous communication (Emmens, 1947), the influence of pH on the motility and viability of rabbit spermatozoa was described in some detail. The investigation was conducted with buffered dextrose solutions at or near the osmotic pressure of physiological saline, under which conditions it was shown that the spermatozoa are more rapidly immobilized and killed in acid than in alkaline media. There was, moreover, a positive correlation between the mean index of motility and the mean percentage mortality between the pH levels of 6 and 10. Below a pH of about 5·8, the spermatozoa are rapidly immobilized and killed, but may remain alive for 1 or 2 hr. in an immotile state. Yamane (1920) has shown that equine spermatozoa live rather longer in hypotonic saline (0·88 %) than in more concentrated solutions, but live longer in isotonic dextrose than in either hypo- or hypertonic dextrose. Milovanov (1934), recording work by himself and other Russian investigators, describes a stimulating effect of electrolytes on motility, but states that too high an electrolyte concentration is harmful and exhausts the spermatozoa. He quotes Pojorkov as postulating that the lower the electrolyte concentration, the lower should be the pH for maximal survival, and the lower the pH, the lower should be the content of electrolytes.

Preliminary observations on rabbit spermatozoa in this laboratory also indicated that variation in the tonicity and electrolyte content of the diluting fluid influences motility in different ways at different hydrogen-ion concentrations. It was therefore decided to investigate this interaction more fully, with diluents containing dextrose and sodium chloride in varying proportions, using the motility index as previously defined. The use of this index avoids several difficulties which occur if an attempt is made to determine the percentage of living spermatozoa by staining methods. It allows of the completion of a test within 6 hr., a period during which the pH of suspensions remains constant; it allows of more observations per test than is possible with the alternative method of making and staining smears for determining percentage mortality, and it permits the greater dilution of ejaculates which is necessary when making many simultaneous observations on the same ejaculate. These conditions are all necessary for the success of the factorial tests described in the present paper.

MATERIAL AND METHODS

Tests were conducted at room temperature as described by Emmens (1947), with up to eighteen tubes per ejaculate, and dilutions in different tests at from 5 to 30 million cells per c.c. Only active ejaculates with high counts were used in the tests. The motility score was usually determined at ½, 1½, 2½, 4 and 5½ hr. from the start of each test, and the added scores for each tube constitute the motility index, which is unit observation in the analysis. The score ranges from 4 at full motility to zero at complete immotility.

Details of the diluents used in each test are given in Table 1. All diluents were dextrose and sodium chloride mixtures buffered with sodium phosphates or bicarbonate-carbonate mixtures. The relative tonicity of a diluent was calculated on the assumption that all electrolytes present were completely dissociated.

TABLE 1. Constitution of diluents

			Relative tonicity (0·9 % NaCl = 100)			
Diluent	% dextrose	% NaCl	pH 6·4	pH 7·2	pH 8·6	pH 9·7
A	3·0	0·39	110	113	109	112
B	3·0	0·13	81	83	80	82
C	4·6	0·13	110	113	109	112
D	3·0	0·65	139	142	138	141
				pH 8·0		
E	2·0	0·0	51	55		52
F	2·0	0·46	100	104		101
G	2·0	0·92	149	153		150
H	1·0	0·17	51	55		52
J	3·7	0·17	100	104		101
K	6·4	0·17	149	153		150

The content of 0·1 M. buffers in diluents A–D was 17 c.c./100 c.c.; in E–K it was 20 c.c./100 c.c. The tonicity of the same diluent varies slightly from pH to pH because of alterations in ionic composition, but the difference in tonicity between diluents is the same for all pH levels. This difference is 29–30 for the A–D series and 49 for the E–K series, when 0·9 % NaCl = 100. Phosphate buffers were used for pH 6·4, 7·2 and 8·0, and bicarbonate-carbonate buffers for pH 8·6 and 9·7. It has already been shown (Emmens, 1947) that these buffers are about equally innocuous to spermatozoa. The nominal pH of the buffers is sometimes altered by the addition of semen, and this occurrence is noted in the relevant tables. Diluents of the same tonicity as 0·9–1 % sodium chloride will be referred to as isotonic, those below and above that tonicity range will be referred to as hypo- and hypertonic respectively.

RESULTS

Preliminary tests. The range over which studies of the effect of tonicity (and electrolyte concentration) might usefully be made was estimated from the test shown in Table 2. The tonicity of Baker's solution was varied in this test by altering the sodium chloride content, and in the most hypotonic modification by diluting a chloride-free solution to 80 % of its concentration. It will be seen that the spermatozoa were by no means rapidly immobilized by tonicities as extreme as 57–158 % of that of physiological saline.

Table 2. The effect of alteration of the sodium chloride content of Baker's solution on the motility of rabbit spermatozoa in vitro. The table gives indices of motility

Time from start of test (hr.)	Tonicity of diluent (0·9 % NaCl = 100)					
	57	71	93	115	137	158
½	3½	3½	4	4	4	3½
1	3½	3½	4	4	3½	3
3	2	2½	3	3½	2½	2
6	1	1	2½	3	2	1½

The next test was factorially designed to explore the effects of mild hypotonicity, pH and protection of the suspension of spermatozoa from contact with the air. The last factor was included because of fears that results might not be applicable to conditions involving the restricted access of air. Diluents A and B were used at four pH levels, their relative tonicities being 110:80 approximately, and duplicate tubes were put up at each pH, one with and one without a layer of 1½–2 cm. of liquid paraffin over the suspension. The motility indices totalled for the whole test as outlined above are given in Table 3. These indices are multiplied by 4 to avoid fractions, as quarter-grades were sometimes used in scoring the motility of individual suspensions. The test was made with a single ejaculate. The analysis of variance for this test is given in Table 4, in which all interactions are formally isolated. It was to be expected that the diluent/pH interaction would be the only one to show significance, but the others have been kept separate, not only to illustrate the satisfactory nature of the test, but also because they might conceivably have been significant, and it

Table 3. Motility indices (total × 4) for a test of the influence of sodium chloride concentration, pH and semi-anaerobic conditions on the motility of rabbit spermatozoa in vitro

	pH of suspension				
Diluent	6·4	7·2	8·2–8·5	9·6–9·8	Total
A	24	74	60	30	188
A + oil cover	25	74	60	32	191
B	18	69	59	42	188
B + oil cover	18	70	60	42	190
Total	85	287	239	146	757

Table 4. Analysis of variance for the data of Table 3

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P*
Oil *v.* no oil	1	1·563	1·563	2·8	>0·05
Diluents (A:B)	1	0·062	0·062	—	—
pH	3	6192·188	2064·063	3669·4	<*0·001*
Interactions					
Oil/diluent	1	0·062	0·062	—	—
Oil/pH	3	0·188	0·062	—	—
Diluent/pH	3	183·687	61·229	108·85	<*0·01*
Residual	3	1·688	0·563		

* The probability that the observed effect is due to chance.

is not, in the author's view, justifiable to combine them with the error term. The second order interaction, oil/diluent/pH, is the residual variance used aserror.

The absence of a significant difference between motilities with the two different diluents is clearly an accident, in that differences between the effect of the two diluents at the particular pH levels chosen happen to balance out when the overall effects are examined. The actual situation is revealed by a highly significant diluent/pH interaction, showing that the dependence of motility on sodium chloride concentration is different at different pH levels. This is seen clearly in Table 3, which shows that, at an acid pH, the motility in this specimen was best in diluent A, whereas at the more extreme alkaline pH it was best in diluent B. The highly significant effect of pH is to be expected, and the absence of a significant effect from an oil cover to the suspensions indicates that this precaution is not necessary.

As an illustration of the course of motility during this test, Table 5 lists the initial (½ hr.) and terminal (5½ hr.) motilities of each suspension.

TABLE 5. The individual ½ hr. and 5½ hr. motility indices in the test summarized in Table 3. The ½ hr. motility index is given first in each cell of the table

	pH of suspension			
Diluent	6·4	7·2	8·2–8·5	9·6–9·8
A	3, 0	4, 3½	4, 2½	4, 0
A + oil cover	3, 0	4, 3½	4, 2½	4, ½
B	2½, 0	4, 3	4, 2½	3½, 1¼
B + oil cover	2½, 0	4, 3¼	4, 2½	3½, 1¼

It is thus seen that, in acid suspension, the spermatozoa were inhibited in motility early in the test, and became immotile by the end; whereas in alkaline suspension, at pH 9·7, they showed little or no inhibition at first, became practically immotile in diluent A, but retained some motility in diluent B, although with this diluent they were rather less active initially. The motility rating at pH 7·2 was well maintained, and was still between 2½ and 3 in all tubes 24 hr. later. This is a rather better maintenance of motility than the average ejaculate exhibits.

More detailed tests. The next test (Table 6) was more comprehensive, consisting in four replications of a factorial scheme; each replication was made with a different ejaculate and on a different day. Diluents A–D were used, each at four pH levels. The test was designed to investigate (*a*) differences between ejaculates, (*b*) the effect of alterations in sodium chloride concentration, (*c*) the effect of replacement of two-thirds of the sodium chloride by dextrose in isotonic solutions, and (*d*) the interactions of these factors with pH. Although the effects of day of testing and the particular ejaculate used cannot be disentangled in analysis, there can be little doubt that the differences observed are due predominantly to ejaculates, as other tests in which more than one ejaculate was used on the same day have shown corresponding

Table 6. Motility indices (total × 4) for the first test of the influence of sodium chloride and dextrose concentrations, pH and different ejaculates on the motility of rabbit spermatozoa in vitro

	pH of suspension																				
	5·8–6·4					7·0–7·3					8·4–8·7					9·6–9·7					
Ejaculates...	1	2	3	4	Total	1	2	3	4	Total	1	2	3	4	Total	1	2	3	4	Total	Totals
Diluent																					
A	20	41	17	17	95	78	71	73	77	299	68	55	65	65	253	46	35	51	44	176	823
B	16	38	12	16	82	73	60	58	70	261	66	47	59	63	235	52	38	52	45	187	765
C	34	43	12	16	105	73	62	68	75	278	66	50	61	69	246	59	35	54	51	199	828
D	32	44	19	21	116	70	60	71	75	276	63	47	55	53	218	41	26	30	32	129	739
Totals	102	166	60	70	398	294	253	270	297	1114	263	199	240	250	952	198	134	187	172	691	3155

differences in activity and response to pH changes. As in Table 3, the motility indices in each cell of the table are the five readings for each tube added together and multiplied by 4. The analysis of variance is shown in Table 7.

Table 7. Analysis of variance for the data of Table 6

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P*
Ejaculates	3	438·5	146·2	11·7	*<0·001*
pH	3	18417·4	6139·1	491·8	*<0·001*
Diluents	3	359·5	119·8	9·6	*<0·001*
Interactions					
Ejaculate/pH	9	2761·6	306·8	24·6	*<0·001*
Ejaculate/diluent	9	107·5	11·9	—	—
pH/diluent	9	859·6	95·5	8·0	*<0·001*
Residual	27	337·0	12·5	—	—

* The probability that the observed effect is due to chance.

This analysis shows that the situation is complex, with highly significant interactions between pH and ejaculates and pH and diluents. As the pH was not constant in suspensions meant to be at the same pH, particularly in the acid group, and since the effect of variation in pH will be different at different pH levels, the test has been examined further by applying covariance analysis within each of the four pH groups separately, using pH as the concomitant variable. The three degrees of freedom associated with the four diluents have also been isolated by factorial analysis according to the scheme:

	Factorial coefficients for diluents			
Source of variation	A	B	C	D
(1) Difference in concentration of NaCl with constant dextrose	0	−1	0	+1
(2) Part replacement of NaCl by dextrose (isotonic)	−1	0	+1	0
(3) Advantage of isotonicity	+1	−1	+1	−1

The first source of variation is the difference in effects of hypertonicity and hypotonicity due to sodium chloride ions. The second is that due to the difference in effect between varying proportions of sodium chloride and dextrose, adjusted in each case, to produce isotonicity. The third is the extent to which isotonicity with 1 % sodium chloride is superior in effect to the average of the two solutions which depart from isotonicity by the same amount but in opposite directions.

The results are given in Table 8. Covariance analysis affected the conclusions only at pH 5·8–6·4, where the first source of variation just discussed would have been judged significant from the crude data, and reduced the error mean square from 17·1 to 11·8. Since no improvement in accuracy or change in conclusions occurred after applying covariance in the other pH groups, these data are presented in Table 8 without application of the technique. These analyses show that (*a*) at pH 5·8–6·4 no significant effects are detectable; (*b*) at pH 7·0–7·3 both the hyper- and hypotonic diluents have an adverse influence on motility, and replacement of most of the sodium chloride by dextrose is slightly unfavourable to motility; (*c*) at pH 8·4–8·7 both hyper- and hypotonicity are again unfavourable, but no significant effect is seen on replacing sodium chloride by dextrose in isotonic diluents; (*d*) at pH 9·6–9·7 hypotonicity (i.e. the withdrawal of sodium chloride) favours motility. The

Table 8. Separate analyses of variance for the data of Table 6 at each pH level, using covariance to correct for variations in the pH of individual tubes in the pH group 5·8–6·4

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio	*P**
		pH 5·8–6·4			
Diluents: Effect 1	1	29·4	29·4	2·5	>0·05
Effect 2	1	32·9	32·9	2·8	>0·05
Effect 3	1	21·6	21·6	1·8	>0·05
Ejaculates	3	1420·0	473·3	40·0	<*0·001*
Residual	8	94·7	11·8		
		pH 7·0–7·3			
Diluents: Effect 1	1	28·1	28·1	2·7	>0·05
Effect 2	1	55·1	55·1	5·4	<*0·05*
Effect 3	1	100·0	100·0	9·8	<*0·05*
Ejaculates	3	326·3	108·8	10·6	<*0·01*
Residual	9	92·3	10·3		
		pH 8·4–8·7			
Diluents: Effect 1	1	36·1	36·1	4·5	>0·05
Effect 2	1	6·1	6·1	—	—
Effect 3	1	132·3	132·3	16·5	<*0·01*
Ejaculates	3	573·5	191·2	23·9	<*0·001*
Residual	9	72·0	8·0		
		pH 9·6–9·7			
Diluents: Effect 1	1	420·5	420·5	29·9	<*0·001*
Effect 2	1	66·1	66·1	4·7	>0·05
Effect 3	1	217·6	217·6	15·5	<*0·01*
Ejaculates	3	585·7	195·2	13·9	<*0·001*
Residual	9	126·6	14·1		

* The probability that the observed effect is due to chance.

replacement of sodium chloride by dextrose in isotonic diluents, while it increased motility in this particular test, did not do so to a statistically significant extent.

The combined results of this and the previous test show that the effect of variation in the sodium chloride content of diluents covering a range of osmotic pressures of 80–140 approx. is small at any given pH; yet within this range a differential action occurs at different pH levels. This is illustrated in Fig. 1, from which it can be seen that the relationship between tonicity and the motility index swings over, as the pH increases, from higher motility at higher osmotic pressures, through a range in which isotonicity is most favourable, to higher motility at lower osmotic pressures. The substitution of dextrose for sodium chloride in isotonic media had little effect at any pH; there was perhaps a tendency for a higher sodium chloride content to be less favourable in alkaline media.

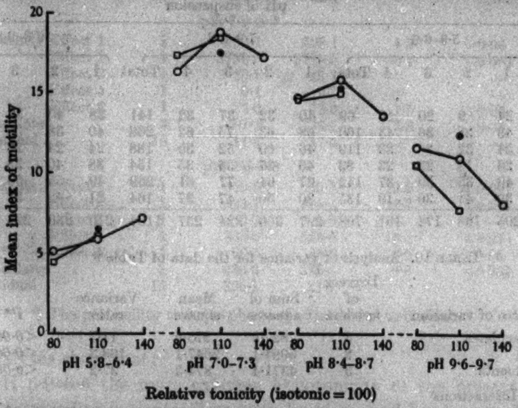

Fig. 1. The interaction of pH and osmotic pressure on the motility of rabbit spermatozoa. The mean indices of motility (see text) are taken from the following tables:

□—□ Table 3 } Constant percentage of dextrose, varying percentage of sodium chloride.
○—○ Table 6 }
• Table 6. Effect of decreasing the sodium chloride content from 0·39 to 0·13 % and replacing by dextrose in isotonic diluents. To be compared with ○—○.

The last test consists in a further investigation of these points. Diluents E–K were used at three pH levels: 6·4, 8·0 and 9·7 approx. The series E–G comprises three diluents with a constant amount of dextrose but increasing amounts of sodium chloride, covering a tonicity range of 50–150 approx., while the series H–K comprises three diluents covering the same range of tonicity in which the dextrose content is varied and the sodium chloride content remains constant. Whereas the first series includes a member with no sodium chloride, the second starts with 1 % dextrose, since the absence of dextrose would rob the spermatozoa of their nutriment. The test was repeated with four ejaculates, and the results are given in Table 9, the individual entries in which were calculated as for Table 6. The analysis of variance is shown in Table 10.

TABLE 9. Motility indices (total × 4) for the second test of the influence of sodium chloride and dextrose concentrations, pH and different ejaculates on the motility of rabbit spermatozoa in vitro

	pH of suspension															
	5·9–6·6					7·9–8·2					9·6–9·8					
Ejaculates...	1	2	3	4	Total	1	2	3	4	Total	1	2	3	4	Total	Totals
Diluent																
E	24	9	20	16	69	40	32	37	32	141	38	40	38	35	151	361
F	43	38	36	43	160	68	63	75	62	268	40	38	38	22	138	566
G	34	32	30	23	119	46	60	52	30	188	24	24	28	13	89	396
H	25	13	22	23	83	46	37	36	35	154	38	40	38	35	151	388
J	40	35	30	37	142	67	64	77	61	269	49	55	50	36	190	601
K	39	41	36	19	135	30	50	47	37	164	21	42	32	16	111	410
Totals	205	168	174	161	708	297	306	324	257	1184	210	239	224	157	830	2722

TABLE 10. Analysis of variance for the data of Table 9

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P*
Ejaculates	3	827·8	275·9	11·7	*<0·001*
pH	2	5094·1	2547·1	108·3	*<0·001*
Diluents	5	4371·4	874·3	37·2	*<0·001*
Interactions					
Ejaculate/pH	6	398·3	66·4	2·8	*<0·05*
Ejaculate/diluent	15	814·0	54·2	2·3	*<0·05*
Diluent/pH	10	2830·2	283·0	12·0	*<0·001*
Residual	30	705·3	23·5	—	—

* The probability that the observed effect is due to chance.

The same considerations as before lead to the splitting of the data into separate groups at each pH. There was no significant improvement on applying covariance analysis to correct for variations in the pH of individual tubes; it is therefore omitted in Table 11, which gives the analyses of variance. In this table, the five degrees of freedom associated with the six diluents have been isolated according to the scheme:

	Factorial coefficients for diluents					
Source of variation	E	F	G	H	J	K
(1) Hypo- *v.* hypertonicity	+1	0	−1	+1	0	−1
(2) Advantage of isotonicity	−1	+2	−1	−1	+2	−1
(3) Glucose *v.* NaCl hypotonic	+1	0	0	−1	0	0
(4) Glucose *v.* NaCl isotonic	0	+1	0	0	−1	0
(5) Glucose *v.* NaCl hypertonic	0	0	+1	0	0	−1

The sources of variation are self-explanatory.

TABLE 11. Separate analyses of variance for the data of Table 9 at each pH level

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P*
		pH 5·9–6·6			
Diluents: Effect 1	1	650·3	650·3	20·3	<*0·001*
Effect 2	1	816·8	816·8	25·4	<*0·001*
Effect 3	1	24·5	24·5	—	—
Effect 4	1	40·5	40·5	1·3	>0·05
Effect 5	1	32·0	32·0	—	—
Ejaculates	3	188·3	62·8	2·0	>0·05
Residual	15	481·7	32·1	—	—
		pH 7·9–8·2			
Diluents: Effect 1	1	203·1	203·1	4·3	>0·05
Effect 2	1	3798·5	3798·5	80·1	<*0·001*
Effect 3	1	20·1	20·1	—	—
Effect 4	1	0·1	0·1	—	—
Effect 5	1	72·0	72·0	1·5	>0·05
Ejaculates	3	401·1	133·7	2·8	>0·05
Residual	15	711·4	47·4	—	—
		pH 9·6–9·8			
Diluents: Effect 1	1	650·3	650·3	29·9	<*0·001*
Effect 2	1	494·1	494·1	22·7	<*0·001*
Effect 3	1	0·0	0·0	—	—
Effect 4	1	338·0	338·0	15·6	<*0·01*
Effect 5	1	60·5	60·5	2·8	>0·05
Ejaculates	3	636·9	212·3	9·8	<*0·001*
Residual	15	326·1	21·7		

* The probability that the observed effect is due to chance.

These analyses show that:

(*a*) At pH 5·9–6·6 the more extreme hypotonicity of the present test is deleterious to motility in comparison with both iso- and hypertonic diluents. No influence of substituting dextrose for part of the sodium chloride is seen.

(*b*) At pH 7·9–8·2 both hypo- and hypertonic diluents are, as before, equally unfavourable in comparison with isotonic media. Again, no overall effect is seen on part substituting dextrose for sodium chloride.

(*c*) At pH 9·6–9·8 hypotonicity is more favourable than hypertonicity, and at these extremes the replacement of sodium chloride by dextrose has no significant effect. In isotonic diluents, however, the replacement of most of the sodium chloride by dextrose enhances motility, and the favourable effect of hypotonicity is seen to be due to removal of part of the sodium chloride rather than hypotonicity *per se*. At this pH, differences between ejaculates, insignificant at the other pH levels, become apparent in this particular test.

A reversal in the relationship between tonicity and pH is seen as in the previous tests, and is illustrated in Fig. 2.

Since motility at the various pH levels covered by this investigation has been shown to be positively correlated with viability (Emmens, 1947), at least under isotonic conditions, the contention of Pojarkov that a low pH requires a low electrolyte concentration for the preservation of spermatozoa is contradicted by these results. It is only at a high pH, in isotonic media, that rabbit spermatozoa do not tolerate a relatively high concentration of sodium chloride.

It will be seen that the responses in the group at pH 5·9–6·6 are higher than in the previous test. The mean pH in this group was 6·34; in the previous test it was 6·16. In this very sensitive region, the slight shift in mean pH is enough to explain the greater part of the discrepancy, for if we assume a linear relationship between pH and motility over the range 6·16–7·14 (the mean pH of the first and second groups respectively in the previous test) the increase in total motility index per ejaculate of isotonic suspensions is 4·8 per 0·1 rise in pH. For diluents A and C, the total index at pH 6·16 was 25·0; we therefore expect a mean index of about 33·7 for the isotonic diluents F and J. The actual figure is 37·8, and the remaining discrepancy is not serious, in view of differences between ejaculates and diluents in the two tests.

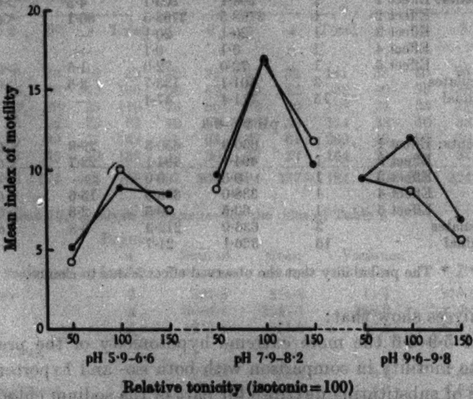

Fig. 2. The interaction of pH, osmotic pressure and electrolyte content of diluents on the motility of rabbit spermatozoa. The mean indices of motility (see text) are taken from Table 9:

o—o Constant percentage of dextrose, varying percentage of sodium chloride.
●—● Constant percentage of sodium chloride, varying percentage of dextrose.

In Table 5, it was seen that the hypotonic diluent B lowered motility below the initial level in alkaline suspension in the one ejaculate there examined, but that motility was maintained better at pH 9·7 by 5½ hr. than with diluent A, which was isotonic. This improved maintenance is again apparent in Tables 12 and 13, which list the mean initial and final (5½ hr.) motility indices in the two large-scale tests.

Table 12. Mean ½ hr. and 5½ hr. motility indices for the four replications in the list of Table 6

The ½ hr. index is given first in each cell of the table

	pH of suspension			
Diluent	5·8–6·4	7·0–7·3	8·4–8·7	9·6–9·7
B. Hypotonic 3 % dextrose	2·4, 0·3	3·9, 2·3	3·6, 2·3	3·3, 1·5
A. Isotonic 3 % dextrose	2·8, 0·3	3·9, 3·3	3·9, 2·7	3·3, 1·1
C. Isotonic 4·6 % dextrose	2·7, 0·4	3·9, 2·8	3·6, 2·5	3·4, 1·6
D. Hypertonic 3 % dextrose	3·1, 0·4	3·8, 2·7	3·8, 1·7	3·0, 0·4

Table 13. Mean ½ hr. and 5½ hr. motility indices for the four replications in the list of Table 9

The ½ hr. index is given first in each cell of the table

	pH of suspension		
Diluent	5·9–6·6	7·9–8·2	9·6–9·8
H. Hypotonic 1 % dextrose	2·4, 0·3	2·4, 1·5	2·3, 1·5
E. Hypotonic 2 % dextrose	2·2, 0·1	2·4, 1·0	2·3, 1·5
F. Isotonic 2 % dextrose	3·5, 0·5	3·8, 2·8	2·9, 0·5
J. Isotonic 3·7 % dextrose	3·5, 0·3	3·8, 3·0	3·1, 1·6
G. Hypertonic 2 % dextrose	3·4, 0·2	3·2, 1·4	2·5, 0·2
K. Hypertonic 6·4 % dextrose	2·9, 0·2	2·2, 1·9	1·7, 1·0

Tables 12 and 13 demonstrate the peculiarities of the interaction between tonicity and pH. Spermatozoa in hypotonic acid diluents (pH 6·2 approx.) are less motile, within half an hour, than those in iso- or hypertonic conditions, whatever the relative content of dextrose and sodium chloride. By 5½ hr., however, the motility of all suspensions has fallen to about the same level. In neutral or mildly alkaline suspension (pH 8 approx.), both hypo- and hypertonic, the spermatozoa are initially less active than those in isotonic suspension (with one exception in Table 12) and continue to lose motility more rapidly, except in the presence of a relative excess of dextrose, which tends to produce a more constant low level of motility throughout the test. In more alkaline hypotonic suspension (pH 9·7 approx.), the spermatozoa are less active initially than in isotonic suspension, but they lose little motility and finish the test with a higher rating than those in isotonic suspension, unless the extra tonicity is contributed by dextrose. This saving effect of dextrose continues into the hypertonic range, where the rate of fall in motility is checked by additional dextrose as compared with additional sodium chloride. The early motility is not, however, improved by this addition.

SUMMARY

1. The motility of rabbit spermatozoa in vitro has been studied at various pH levels with diluents of different tonicity and chemical constitution. Tonicities between that of 0·45 and 1·35 % sodium chloride have been obtained by alteration in the sodium chloride content and/or the dextrose content of the diluents. These diluents were buffered with sodium phosphate or carbonate-bicarbonate systems.

2. At pH 5·8–6·6, a region in which rabbit spermatozoa are less motile than at pH 7–8 and are very sensitive to pH changes, they are more sensitive to hypotonicity than to hypertonicity, and are affected in motility little or not at all by substitution of part or all of the sodium chloride by dextrose.

3. At pH 7·0–8·7, a region in which the spermatozoa are relatively insensitive to changes in pH, hypo- and hypertonicity, whether caused by alteration in sodium chloride or dextrose concentration, are about equally deleterious to motility. The replacement of sodium chloride by dextrose in hypertonic diluents, however, tends to lower initial motility but to maintain this level of motility with little fall over the next 6 hr.

4. At pH 9·6–9·8, a region in which the spermatozoa are again markedly less motile than at pH 7–8, but are still not very sensitive to minor changes in pH, hypertonicity is more deleterious than hypotonicity, which may even be favourable to motility under certain conditions. The replacement of sodium chloride by dextrose is favourable to motility in isotonic media, but makes little difference in hypo- or hypertonic conditions. The favourable effect of hypotonicity is exerted if it involves a reduction in the sodium chloride content of the diluent, but not if it involves a reduction in dextrose content. The characteristic action of dextrose is, as at pH 7·0–8·7, a lowering of the rate of fall of motility, not the maintenance of a high initial motility.

5. Thus, in alkaline suspension, rabbit spermatozoa are adversely affected by a sodium chloride concentration of over about 0·2 %, remaining more motile in a hypotonic medium of less than this sodium chloride content than in an isotonic medium if it includes more than this amount of sodium chloride.

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THE DISTRIBUTION OF REDUCING SUBSTANCES BETWEEN THE INTRA-OCULAR FLUIDS AND BLOOD PLASMA, AND THE KINETICS OF PENETRATION OF VARIOUS SUGARS INTO THESE FLUIDS

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The concentration of reducing substances (hereinafter referred to as glucose) in the aqueous humour is less than that in plasma (Adler, 1930; O'Brien & Salit, 1931); the concentration in the vitreous body is still smaller. In view of the tendency to ascribe differences in concentration between plasma and intra-ocular fluids to secretory activity it is important that the factors determining these differences be completely elucidated.

We may expect the relative concentrations of diffusible substances in plasma and intra-ocular fluid to be determined by three principal influences:

(*a*) The physico-chemical factors determining the relative compositions of a protein-containing solution in contact with its ultra-filtrate dialysate. If these factors operated alone, non-electrolytes would be equally distributed between the two fluids.* The concentrations of diffusible ions would conform to the Gibbs-Donnan relationship, the cations being more strongly concentrated in the plasma.

(*b*) The metabolism of the eye. The retina and the lens have an active metabolism and in so far as they derive materials directly from the intra-ocular fluids or modify the capillary blood concentration in the uveal tract, this metabolism must influence the observed distribution of solute between arterial blood plasma and intra-ocular fluids. We may expect the effect to be most pronounced on a non-electrolyte, like glucose, but the ionic equilibria can also be modified secondarily.

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* That is, their *activities* should be the same in the two fluids. The observed concentrations need not necessarily be equal; thus the work of Weber & Nachmannsohn (1929) indicates that the concentration of glucose in plasma may be some 2% higher than that in its ultra-filtrate. When concentrations of a substance are compared in a protein-containing and protein-free solution it is important that they be expressed in terms of the weight of water in the solution rather than the volume of the latter; except where otherwise stated, 'concentration' in this paper means weight of substance in 100 g. H_2O.

(c) Secretory activity of the lining membranes. Our knowledge of the contribution of this factor is scanty, although the work of Friedenwald and his collaborators (e.g. Friedenwald & Stiehler, 1938; Friedenwald & Buschke, 1941 and Friedenwald, Buschke & Michel, 1943) is suggestive. Until the simpler physico-chemical and metabolic factors are completely elucidated, little definite can be stated.

In the present paper, the possible effects of these factors on the distribution of glucose between the intra-ocular fluids and blood plasma will be described; the experimental approach has been predominantly by way of kinetic studies, and these have led to tentative conclusions regarding the site of entry of dissolved substances into the eye, and the nature of the membranes separating the aqueous humour and vitreous body from the blood plasma.

EXPERIMENTAL

Static Studies. Great care was taken to avoid the hyperglycaemia associated with fear; nembutal was used as anaesthetic, intraperitoneally in the cat and intravenously in the rabbit. In the latter animal it is impossible to avoid hyperglycaemia, and before fluids were removed the animal was 'equilibrated' for some hours with its head projecting from a bleeding-box. For comparison of normal and aphakic eyes this equilibration was not so important and was generally omitted. Blood was withdrawn from the cat by arterial puncture, and from the marginal ear vein of the rabbit after vaso-dilatation with toluene. Sugars were determined in duplicate by the Hagedorn-Jensen technique (1923) on Somogyi (1930) filtrates of the various fluids; sucrose was determined by estimating the reducing value of the fluid before and after hydrolysis in 0·1 N-H_2SO_4 (15 min. at 100° C.). The difference between duplicates rarely exceeded 1%, except if the reducing value was low when it amounted to 2%. Removal of the lens was carried out by the standard surgical procedure for extra-capsular extraction.

Dynamic Studies. The general principle was to maintain a constant high level of a given sugar in the blood by continuous intravenous infusion of an isotonic solution in 6% gum acacia under nembutal anaesthesia. Rapid elimination of the injected sugar was prevented by tying the renal arteries. One eye was, in general, removed before the injection, to estimate the initial reducing value of the intra-ocular fluids, and the other after a known time interval. With sucrose, which is estimated independently of the reducing substances in the fluids, the preliminary removal of one eye is unnecessary. After enucleation, the aqueous humour was withdrawn and the eye was then frozen in solid CO_2. When hard, the eye was cut into two or more pieces as described later; the frozen pieces of vitreous body were filtered through glass-wool as they melted, in order to break down the gel structure. Blood and aqueous humour were immediately placed in tubes surrounded with ice.

RESULTS

The normal distribution. The concentrations of glucose in the aqueous humour, vitreous body, and blood plasma of rabbits and cats are shown in Table 1. The results on the cat amply confirm those of Adler (1930); the low glucose content of the vitreous body is striking.

There are two main loci of sugar utilization in the eye—the lens and retina. That the lens modifies the sugar content of both the aqueous and vitreous humours is shown in Table 2, in which some typical results on rabbits and cats are presented. The lens was removed from one eye, and at various intervals

TABLE 1. Glucose concentrations (mg./100 g. H_2O) in plasma and intra-ocular fluids

		Fluid			
	Plasma	Aqueous	Vitreous	R_A = aq./pl.	R_V = vitr./pl.
Rabbit	131	116	56	0·89	0·425
	133	118	66	0·89	0·495
	142	110	74	0·78	0·52
	149	128	87	0·86	0·58
	160	134	73	0·84	0·46
	163	145	82	0·89	0·50
Mean				0·86	0·49
Cat	125	95	64	0·76	0·51
	89	73	55	0·82	0·615
	92	78	52	0·85	0·565
	119	86	61	0·72	0·51
	104	67	44	0·64	0·425
	121	89	58	0·74	0·48
	103	89	64	0·87	0·62
Mean				0·77	0·53

TABLE 2. Glucose concentrations (mg./100 g. H_2O) in fluids of normal and aphakic eyes

			Fluid			
	Interval after extraction (weeks)	Plasma	Normal aqueous	Aphakic aqueous	Normal vitreous	Aphakic vitreous
Cat no. 1	4	89	64·5	83	—	—
	6	98	72	83	—	—
Cat no. 2	7	88	66·5	81	—	—
	9	104	66·5	83	44	57
Cat no. 3	3	95	76	81	—	—
	4	—	74	81	—	—
	6	88	76	82	45	53
Rabbit no. 1	3	214	165	149	—	—
	5	183	127	99	—	—
	6	185	166	147	—	—
	7	208	157	129	99	107
Rabbit no. 2	3	161	152	145	—	—
	5	126	103	105	—	—
	6	145	126	121	—	—
	7	178	162	158	99	111
Mean (9 rabbits), 7–14 weeks:		152	124	117	87	96

after the operation aqueous humour was withdrawn from both; finally, both eyes were enucleated. In the cat, the increase in the aqueous humour concentration after lens extraction was pronounced (20% or more) in two cases, whilst in the rabbit it was much smaller; in fact, there may be an actual *decrease*, as in rabbit no. 1. This seems to be due to diffusion backwards through the pupil into the vitreous body, since rabbit no. 1, in which this effect was most marked, had a wide iridectomy and dilated pupil, whereas rabbit no. 2 had no iridectomy, a constricted pupil, and a portion of dead lens partially covering the aperture. Similarly cats nos. 1 and 2 had portions of dead lens material remaining in the pupil whilst in cat no. 3 the pupil was wide and completely unobstructed. The mean for nine rabbits shows a 6% decrease in the aqueous humour of the aphakic eye. The vitreous body showed a large increase in the cat (30%) and a smaller one in the rabbit. The normal eye of the rabbit contains a higher concentration of ascorbic acid than the aphakic eye (Goldman & Buschke, 1935) and, since this substance contributes to the reducing value of the aqueous humour, the increase in glucose concentration due to aphakia is partially masked by the decrease in ascorbic acid concentration.

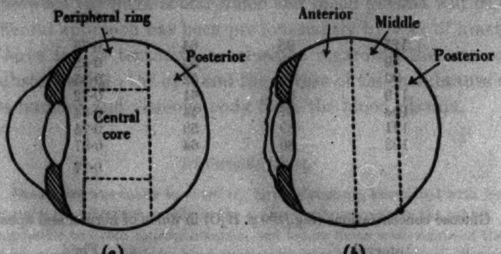

Fig. 1. (a) Approximate sectioning of the vitreous body before and after incubation. (b) Approximate sectioning of vitreous body before and after diffusion experiments; in this case the aqueous humour was removed before freezing.

The fact that the fluids of the aphakic eye do not show sugar concentrations equal to that in plasma suggests that the retina is utilizing glucose. If glucose is consumed by the retina of the enucleated eye, the concentration of glucose after incubation should be less in the periphery than in the centre of the vitreous body. The two eyes of a cat were removed; one was frozen immediately, whilst the other was placed in a boiling tube in the bottom of which there was cotton-wool moistened with 0·9% NaCl; the tube was immersed in a bath at 38–40° C. After 2 hr. the eye was frozen. The eyes were sectioned as indicated in Fig. 1 (a), giving a large central disk and a thinner posterior cap of vitreous body. With a cork-borer the central disk was divided into a core and peripheral ring, the latter about 3 mm. wide. The differences in the glucose contents of the aqueous humour and of the various parts of the vitreous body before and after incubation were as follows:

		mg./100 g.
Vitreous body	Core of central disk	15
	Periphery of central disk	40
	Posterior cap	30
Aqueous humour		27

The result leaves little doubt that the retina is responsible for at least a part of the glucose consumption in the posterior cavity of the eye; if the lens only were consuming glucose, the core of the central disk would have shown the greatest loss since it was in immediate contact with the lens. In the eye, immediately after enucleation, the glucose is apparently not uniformly distributed through the vitreous body; Adler (1930) has shown that in the frozen eye the anterior half of the vitreous body contains more sugar than the posterior. In a series of nine rabbits we have found that if the middle disk (Fig. 1 (*a*)) of the frozen vitreous body was divided into a central core and a peripheral ring, the latter invariably contained the higher glucose concentration. It is possible that these differences in concentration are, at least in part, artefacts resulting from the freezing process, so that it is not justifiable to draw definite conclusions from the observed concentrations regarding the relative extents to which glucose is utilized in the different parts of the eye. The lower concentration in the central core is, however, reasonable, since it is in immediate contact with the lens; on incubation, this relationship is invariably reversed, both in the cat and rabbit; in these circumstances the retina draws on the glucose in the vitreous body owing to the absence of retinal and uveal circulation, and this upsets the normal picture of glucose distribution.

If the lens consumes glucose from the anterior chamber, it may be expected that by reducing the area of lens exposed to the aqueous humour, by miosis, the glucose content should rise. One eye of a cat was treated with eserine and the other with atropine; after about 2 hr. the aqueous humours were withdrawn from both eyes. The mean results for four cats (two of them kittens) were as follows:

	mg./100 g.
Atropinized eye	70
Eserinized eye	75
Plasma	107

Again, it may be expected that the aqueous humour formed immediately after the emptying of the anterior chamber (paracentesis) should have a higher concentration of glucose since the lens will not have had time to re-establish a steady state. The results of a typical experiment are as follows, the 'second' re-formed aqueous humour being the fluid formed after the successive withdrawals of the original and 'first' re-formed aqueous humour:

	mg./100 g. H_2O
Normal aqueous humour	62·5
First re-formed humour	83·5
Second re-formed humour	85·5
Plasma	89

In no instance, however, did the glucose concentration in the re-formed fluid equal that in the plasma exactly.

Kinetic Studies. The permeability of the eye membranes to the following sugars was investigated: glucose, galactose, 3-methyl glucose, xylose and sucrose. 3-Methyl glucose is a synthetic compound which is apparently not phosphorylated in vivo (Campbell, unpublished) and was thought, on this account, to be unlikely to be secreted.

Detailed results of seven experiments with glucose are presented in Table 3 in order that the animal-to-animal variations may be appreciated. Constants indicating the rate of penetration into both aqueous humour (K_A) and vitreous body (K_V) have been calculated in accordance with the equation of Davson & Quilliam (1940).*

TABLE 3. The rate of penetration of glucose into the aqueous humour and vitreous body of the cat

Plasma (mg./100 ml.)	Aqueous (mg./100 ml.) A_1	Aqueous (mg./100 ml.) A_2	Time (hr.)	$100K_A$	$100K_V$	K_A/K_V
280	94	254	2·0	38	11	3·45
290	140	188	0·5	33	16·5	2·0
265	92·5	144	0·75	20·5	12	1·7
266	130	232	2·0	30	10	3·0
280	104	202	0·62	57	7	8·1
597	112	238	0·47	31	10	3·1
439	88	233	0·75	30·5	9	3·4
Mean				34	11	3·1

A_1 is the initial concentration in the aqueous humour; A_2 the concentration in the aqueous humour after the blood concentration, P, had been maintained for t hours. K_A is given by the equation:

$$K_A = \frac{kA}{V} \times \frac{1}{2 \cdot 303} = \frac{1}{t} \log \frac{P - A_1}{P - A_2}$$

where k is the true permeability constant, and A and V are the area and volume of the system respectively. K_V represents a similar constant derived from the concentrations in the vitreous body.

TABLE 4. Penetration of different sugars into the eye fluids of cats

Sugar	No. of cats	$100K_A$	$100K_V$	K_A/K_V	P_S
Glucose	7	34 ±2·2	11 ±0·8	3·1	0·125
Galactose	9	33·5±2·3	9·5 ±0·9	3·5	0·006
3-Methyl glucose	5	36·5±2·9	11 ±0·5	3·3	0·02
Xylose	6	37 ±2·7	16·5 ±1·5	2·2	—
Sucrose	9	4·9±0·6	0·29±0·05	17	—

K_A and K_V have the same meaning as for Table 3. P_S is the probability that the value of Student's t, computed from the mean xylose K_A/K_V and the hexose K_A/K_V, would occur by chance (Yule & Kendall, 1940). Standard errors are also shown.

Table 3 shows a consistent difference in rates of penetration into the aqueous humour and vitreous body, the rate for the aqueous humour being some three times greater. In Table 4 the results for the remaining sugars are presented in the form of the constants, K_A and K_V, only. The following points may be noted: (*a*) The rates of penetration of the pentose (xylose) and of the hexoses into the aqueous humour are not significantly different; the disaccharide, sucrose, on the other hand, penetrates very much more slowly. (*b*) The rates of penetration of the hexoses into the vitreous are all about one-third of those into the aqueous humour ($K_A/K_V = 3{\cdot}1 - 3{\cdot}5$); with sucrose the factor is one-seventeenth ($K_A/K_V = 17$). With the pentose, xylose, the ratio was 2·2, due to an increased value of K_V. Owing to the impossibility of obtaining further supplies of this sugar no more than six experiments could be carried out; consequently Student's '*t*-test' for the differences between means of small samples was applied successively to the mean values of K_A/K_V for xylose and the individual hexoses; the values of P_S (Yule & Kendall, 1940), indicating the probability that the values of *t* so computed would occur by chance, are included in the table. With 3-methyl glucose and galactose the differences in the ratios are significant; with glucose, where greater variability was encountered, the difference is not significant. The large increase of the ratio K_A/K_V on passing to the disaccharide is clearly significant.

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* The equation is only approximate, especially owing to the utilization of sugar by the eye, but in view of the large animal-to-animal variations refinements seem unnecessary. When the plasma reducing value is raised by several hundred % the steady state finally achieved tends to equality of concentration expressed as mg./100 ml. as opposed to mg./100 g. H_2O (i.e. the true concentration in the plasma is about 7% higher than in the aqueous humour), and for this reason the former unit has been used in these kinetic studies.

The results suggest that the membrane separating the vitreous body from the blood plasma is more selective than that separating the aqueous humour; thus the latter apparently does not discriminate between a hexose and pentose, whereas the former appears to do so; as the molecular size is increased, the greater selectivity of the barrier separating the vitreous body from the blood is reflected in an increasing value of K_A/K_V.

The differing values of K_A and K_V for any given sugar suggested that, contrary to accepted belief, substances could enter the eye from other parts than the ciliary body; if penetration takes place only from the ciliary body it would be expected that after maintaining the blood sugar level at a high value for, say an hour, the concentration in the part of the vitreous body most remote from the ciliary body would be smaller than in that closest. In thirteen different experiments, the eyes, after removal of the aqueous humours, and freezing, were cut approximately as in Fig. 1(*b*), giving as mean volumes for the various sections: posterior 0·35 ml., middle 0·43 ml., and anterior 0·63 ml. The different parts were analysed and the results are shown in Table 5. It will be seen that, for the control eyes, the mean concentration in the middle section was 67 mg./100 g. H_2O, being 92% of that in the posterior section; after a high blood-sugar had been maintained for about 1 hr. the average concentration in the middle section was 98 mg./100 g. H_2O, being only 78·5% of that in the posterior section. The results certainly suggest that the locus of diffusion into

TABLE 5. Concentrations of sugar (mg./100 g. H_2O) in three sections of vitreous body after maintaining a high blood concentration for a definite period, compared with the concentrations in the control eye. Mean results of thirteen experiments

	Section of vitreous body		
	Posterior	Middle	Anterior
Control	73	67	75
Test	124	98	113

the vitreous body is not confined to the ciliary body, but it must be remembered that deductions from the concentrations of dissolved material in the frozen eye are to be accepted with caution; conditions of freezing were, however, kept as uniform as possible so that disturbances in distribution would operate to the same extent in control and test eyes.

These results prompted an inquiry into the site of entry of substances into the aqueous humour; if diffusion takes place entirely from the ciliary body, they must enter through the pupil and a variation in the size of the latter might modify the rate of appearance. Diffusion experiments were carried out as before, but one eye of the cat was eserinized and the other atropinized. If diffusion takes place only by way of the ciliary body the rate of entry should be less in the eserinized eye with its slit-like pupil. The results are shown in Table 6; invariably the eserinized eye contained the greater amount of sugar, although the effects were, in general, not large. The results strongly suggest that the pupil size is of no importance in so far as rate of penetration of sugars into the aqueous humour is concerned. The increased rate of penetration into the eye with the constricted pupil is most probably due to an increased capillary permeability resulting from direct action of the drug on the vessels and possibly also a mechanical stretching, since when both galactose and sucrose were studied in the same animal (the bracketed results in Table 6) the effect was much more pronounced on the larger molecule; moreover the eserinized eye generally, but not always, contained a trace of protein.

TABLE 6. The rates of entry of sugars into atropinized and eserinized eyes compared

Sugar	Time (min.)	Atropinized eye	Eserinized eye
Galactose	40	152	214
Galactose	27	215	239
Glucose	60	242	257
Xylose	41	191	199
Galactose }	62	150	164
Sucrose }		18	28
Sucrose	110	38	51

Figures represent sugar concentration in the aqueous humour, in mg./100 g. H_2O, after maintaining a high sugar concentration in the blood for a given time.

DISCUSSION

The membranes lining the eye are readily permeable to hexoses and we may therefore expect that, if physical factors of diffusion alone were operative, the concentrations of glucose in the ocular fluids and the capillary plasma would be equal. The large discrepancy between the concentrations in plasma and ocular fluids has been shown to be primarily due to metabolic influences, and it may well be asked whether the factor of active secretion need be invoked at all. The influence of the lens can be studied in aphakic eyes, and in these the aqueous humour and plasma levels still differ by a factor of at least 10% in the cat. The activity of the retina cannot be excluded, however, and it may well be that removal of the lens permits a ready diffusion of glucose from the anterior chamber into the vitreous body. The experiments with rabbits strongly support this view. The immediately re-formed aqueous humour, after paracentesis, has a glucose concentration differing by only about 5% from that of plasma; here the effects of lens activity and diffusion through the pupil are minimized, and one might expect to find the concentrations equal. Two other factors must, however, be taken into consideration. The blood used for comparison is arterial; the eye is an organ with a very high metabolism and we may therefore expect an arterio-venous difference in sugar concentration (differences as large as 39 mg./100 g. between carotid artery and vortex vein have been reported; the greater portion of this discrepancy is probably due, however, to activity in the more posterior parts of the eye). The glucose concentration in the capillary plasma may therefore be, on the average, less than that in arterial plasma. Again, the non-sugar reducing substances in the plasma represent a considerable fraction of the whole (27 mg./100 ml. according to Somogyi, 1927); the membrane separating the aqueous humour from the plasma has a high degree of selectivity in respect to nitrogenous compounds (unpublished), and it is possible that the re-formed aqueous humour, formed by a rapid process of ultra-filtration and therefore not necessarily equivalent to a dialysate, contains a lower concentration of non-sugar reducing substances than would be the case if time were permitted for dialysis to proceed to completion. The behaviour of sucrose is a case in point; if a high sucrose concentration is maintained in the blood of the cat, it is found that the re-formed aqueous humour after paracentesis has a sucrose concentration only 60% of that in the blood.

The concentration of glucose in the aqueous humour may thus attain a 'steady state' in which the lens consumption is made good by diffusion through the anterior surface of the iris; it is possible to calculate, from the observed concentrations and the constant, K_A, for glucose, the rate of absorption of glucose from the anterior chamber; it amounts to about 0·15 mg./hr., a figure not inconsistent with the known sugar utilization of the lens (Kronfeld & Bothman, 1928).

From the posterior cavity of the eye the lens and retina may both extract glucose, and the concentration in the vitreous body is therefore less than in the aqueous humour. The regional distribution of glucose in the vitreous body is explained by this dual consumption.

The kinetic studies tend to confirm this view of the steady state; the barrier between the plasma and the aqueous humour shows no special selectivity towards glucose but permits galactose, the synthetic 3-methyl glucose, and the pentose, xylose, to pass at about the same rate. If any secretory activity were involved, one might expect, by analogy with intestinal absorption, to find appreciable differences in rates. The slow rate of penetration of sucrose is in agreement with some earlier preliminary work on the dog (Weld, Feindel & Davson, 1942) in which it was shown that this molecule represents the limiting size for sugar penetration, the trisaccharide, raffinose, not penetrating at all.

The striking difference in selectivity between the barriers separating aqueous humour and vitreous body from the blood is of some significance; hitherto it has been considered sufficient, in the interpretation of intra-ocular dynamics, to study the penetration of substances into the aqueous humour only. The existence of this more selective membrane lining the posterior cavity of the eye may well mean that it is the composition of the vitreous body that predominantly determines the intra-ocular pressure; if there is any secretion into the eye it will obviously be more effective in modifying the osmotic pressure difference between the ocular fluid and plasma if the secreted substance does not leak away very rapidly.

It may reasonably be asked whether the differences in K_A and K_V represent actual differences in permeability constants. The constants K_A and K_V contain the factor A/V, the ratio of area of membrane to volume of fluid, and if this is not the same for the two compartments of the eye the constants are not directly comparable. In the cat, the volumes of aqueous humour and vitreous body are roughly in the ratio of 1:1·5, but it is impossible to state the effective areas of membrane; in view of the large number of ciliary processes, it would appear that the area available for diffusion into the vitreous body is considerably the greater, in which case the difference between the true permeability constants would be greater still. However, it is not necessary to know the values of A/V in determining whether the differences in K_A and K_V represent differences in permeability constants; if the membranes had identical characteristics we should expect the ratio K_A/K_V to be the same for different molecules; as we have seen, this is by no means true, the ratio increasing with increasing size of molecule. In a similar way the effects of different viscosity and different rates of mixing in the two fluids should manifest themselves to approximately the same extent for different molecules.

It is of interest to assess the actual value of k, the true permeability constant for hexoses, from an assumed area-to-volume relationship. k is given by:

$$k=\frac{KV}{A}\times 2\cdot 3 \text{ (Davson \& Quilliam, 1940).}$$

Conventionally, k is expressed as the number of g.-mol. of the substance penetrating $1\mu^2$ of surface in 1 sec. when the concentration difference is 1 mol./l. If the volume of the aqueous humour is taken as 1 ml. and the diffusing surface as 1 cm.^{2}, k comes out at approximately 2×10^{-15}. This compares with a value of

$7{\cdot}8 \times 10^{-16}$ for the penetration of urea into the ox erythrocyte, an instance of high permeability. The effective area of diffusing surface for the anterior chamber is, of course, likely to be very much higher than 1 cm.^{2}; if the area-to-volume relationship were comparable with that in the erythrocyte where 1 ml. of cells has an area of about $1 \times 10^{12}\mu^2$ (Ponder, 1934), k becomes 2×10^{-19}. Such an extreme instance of area-to-volume relationship is unlikely to apply to the eye, but even this value of k suggests that the rate of penetration is comparatively high when it is appreciated that the penetration of the smaller molecule, erythritol, into plant cells gives constants ranging from 7×10^{-21} to $1{\cdot}3 \times 10^{-19}$ (vide Davson & Danielli, 1943). It therefore seems likely that the penetration of hexoses into the eye is a rapid permeability process; this fact, and the absence of a significant difference in rate between hexoses and the pentose, might suggest a penetration by way of water-filled pores, e.g. through the intercellular spaces of the iris capillaries and iris endothelium, but evidence derived from a study of the penetration of some other substances is against this simple mechanism and it is more probable that the comparatively rapid rate of penetration of sugars is an instance of cell membrane specialization of the kind described by Davson & Reiner (1942).

It is common, in describing studies on the intra-ocular fluids, to discuss them in relation to dialysis or secretion theories; we are convinced that this sort of discussion is now unprofitable; the problem, as indicated in the introduction to this paper, is to study each constituent of the intra-ocular fluids and to determine whether its concentration and the dynamics of its penetration are in accordance with simple diffusion theory, and next to determine the influence of the internal metabolism of the eye on these factors. Only when these physico-chemical and metabolic factors fail to explain the observed facts does it become necessary to invoke specific secretory activity.

SUMMARY

1. In the cat and rabbit, the glucose concentrations in the aqueous humour and vitreous body are lower than would be expected on the basis of a simple diffusion equilibrium with the plasma. Experiments indicate that the metabolism of both the lens and retina causes this glucose deficit.

2. The rates of penetration of glucose, galactose, 3-methyl glucose, xylose and sucrose into both aqueous humour and vitreous body of the cat's eye have been measured, and constants, K_A and K_V, proportional to these rates, have been calculated. No significant differences in rate of penetration into the aqueous humour were found with the first four sugars, but sucrose penetrated much more slowly. With the vitreous body the penetration rates were consistently smaller, and an analysis of the results indicates the existence of a more selective barrier to diffusion into this part of the eye than that separating the aqueous humour from the blood. The possible physiological significance of this

difference is discussed and the importance of including the vitreous body in any study of intra-ocular dynamics is stressed.

3. The rate of penetration of sugars into the aqueous humour seems to be independent of pupil size and it seems likely that the bulk of diffusion into the anterior chamber takes place from the iris. Some evidence is presented which suggests that the locus of diffusion into the vitreous body is not confined to the ciliary body.

4. By assuming limiting values for the area-to-volume relationship for the aqueous humour, it is possible to calculate the true permeability constants for the hexoses in the conventional units; the computation suggests that penetration is rapid in comparison with that of analogous, lipoid-insoluble, molecules diffusing into plant cells.

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THE ACTION OF CARBON DIOXIDE AND CYANIDE ON FOETAL RESPIRATORY MOVEMENTS; THE DEVELOPMENT OF CHEMOREFLEX FUNCTION IN SHEEP

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In the sheep foetus, rhythmical movements of a respiratory nature are seen from the 40th day onwards. They can be elicited by various afferent stimuli. From the 50th day onwards they can be initiated by oxygen-want also. The anoxic initiation of the respiration is probably a release of the respiratory centre from the control of 'higher' inhibiting centres, for the same picture can be obtained by sectioning the central nervous system (C.N.S.) at various levels (Barcroft & Barron, 1936, 1937, 1939, 1942; Barcroft, Barron, Cowie & Forsham, 1940; Barcroft, 1946).

Snyder & Rosenfeld (1937) and Rosenfeld & Snyder (1938) compared the effect of CO_2 and anoxia on the respiratory movements of rabbit foetuses and of newborn rabbits. From the 28th day onwards the foetuses often exhibited 'spontaneous' respiratory movements which could be depressed by anoxia and acapnia, but often were slightly stimulated by CO_2 in excess. On the other hand, the respiration of newborn rabbits, even of those prematurely delivered, from the foetal age of 29 days onwards, was stimulated by anoxia and CO_2, and by cyanide from the 28th day. The interpretation given by the authors was that the carotid body is capable of its chemoreceptor function from the 28th or the 29th day, but, before birth, there is inhibition of this function and that, in addition, the foetal respiratory centre is less sensitive to excess CO_2 than that of the adult.

Windle, Monnier & Steele (1938) have reported experiments in which both anoxia and CO_2 have given rise to respiratory movements in cat foetuses. Foetuses younger than 40 days regularly gave a response, whereas at term no stimulation of the respiration could be obtained by either CO_2 or oxygen-want. The authors connected this failure with the foetal anoxia which gradually increases towards full term. In sheep, there have been a few observations showing that CO_2 in excess can stimulate the foetal respiration; and it was noteworthy that the respiratory response was rather different from that obtained by anoxia (Barcroft, 1942).

In the present work, an attempt has been made to observe the response of sheep foetuses to an excess of CO_2, from early midfoetal life until term. In adult animals, CO_2 is known to stimulate both the peripheral chemoreceptors and the respiratory centre (Schmidt & Comroe, 1940). In order to get further information about the nature of the stimulation by CO_2, the effect of cyanide which—at least in adult animals—stimulates the respiration only through the chemoreceptor reflexes (Heymans, Bouckaert & Dautrebande, 1932; Wright, 1935), was also studied.

METHODS

The series consisted of twenty-three experiments performed on twenty-two foetuses and on one lamb. Welsh sheep were used. The dates of the tupping were known, and hence the foetal ages. These ages agreed with those found from the tables of age and crown-rump length given by Barcroft (1946).

The ewes were anaesthetized either with intravenous chloral hydrate, or by a spinal anaesthetic, or by a combination of both. Chloral hydrate was used as a 10% solution. It alone was given to eight ewes (668–678, 703), and the dose required varied between 3·5 and 7·0 g. The spinal anaesthetic alone ('duracaine', 1·5–3·0 c.c.) was given to five ewes (667, 700–702, 704). Some of these ewes exhibited a considerable spontaneous hyperventilation. This was avoided, if the combined anaesthesia was used. Another advantage of the combination was that the dose of chloral hydrate could be reduced to 2·0–4·0 g., and the foetal brain was therefore more likely to exhibit its normal behaviour. In adult animals (decerebrated dogs and cats) chloral hydrate is known to decrease slightly the sensitivity of the respiration to CO_2, whereas the sensitivity to cyanide is slightly increased or remains constant (Dripps & Dumke, 1943). The combined anaesthesia was given to seven ewes (691–699).

Examination of the experimental results shows that the use of different anaesthetics was of very little significance. The foetuses were brought under observation by performing a Caesarean section on the ewe. The operation was carried out in a saline bath at body temperature. The uterus was opened, but the amniotic sac was kept whole until injections into the umbilical vessels were needed. The movements of the foetus were observed through the transparent membrane.

Gas mixtures were administered to the mother through a tracheal cannula. If the ewe had had only a spinal anaesthetic, the cannula was inserted under local anaesthesia. To study excess CO_2, the ewe rebreathed from a big Douglas bag containing 600 l. of a CO_2-air mixture. The CO_2 concentration used in the experiments varied between 5·75 and 8·4%, generally being between 7 and 8%. The respiration of the mother was recorded by using a rubber bellows connected through a side tube to the tracheal cannula. The ewe reacted to the CO_2 with a violent hyperventilation. In two ewes the CO_2 concentration of the arterial blood was measured; it rose as a consequence of the CO_2 administration from 43·6 to 46·2 vol. %, and from 59·3 to 65·8 vol. %, respectively. It was concluded that the expected rise of the CO_2 pressure on the foetal side of the placental barrier occurred, first, because of the flushing of the foetal skin, and second, because the pulse rate and the behaviour of the foetus changed. The O_2-poor mixtures used in four experiments contained 5·0–5·7% oxygen and less than 2·2% CO_2 in nitrogen. They, also, were given from Douglas bags containing 60–80 l. of the mixture. The rebreathing always lasted only for a very short time (1½–4 min.) because the respiration of the mother soon failed.

The cyanide was injected as 0·2 mg. %, 1·33 mg. % or 0·133% KCN solution. The solution was injected into the umbilical veins, except in the foetus of 132 days (692), where a carotid artery was used.

The primary aim of the experiments was to observe the development of the respiratory reflexes. In addition, pulse counts were taken, but only when this could be done without undue interference to the foetus or to the umbilical cord.

RESULTS

Chemosensitivity of foetus

The response of the foetus to CO_2 and to cyanide varied with the foetal age. The results are treated as divided into four age groups each of which showed a more or less consistent behaviour.

Respiratory movements not induced. The youngest age group consists of three foetuses: 43 (667, spinal), 46 (672, chloral) and 49 (668, chloral) days old respectively. The first two did not show any spontaneous movements nor did they react to touch. Their lack of motility does not quite fit the general picture of the functional development of the C.N.S. of the sheep foetus (Barcroft, 1946). CO_2 was administered to the mothers of both, and 13·3μg. cyanide to the older foetus. Neither induced any foetal movements.

The foetus of 49 days showed spontaneous somatic movements, as well as spontaneous respiratory rhythms of about ten excursions each. The frequency of the respiratory movements was 46 per min. The somatic reflexes were easily elicited; the foetus reacted vigorously to tapping the sac. Nevertheless, administering CO_2 had no effect on the foetal respiratory movements, nor did tying the umbilical cord.

Respiratory movements induced. The second age group comprises five foetuses: 58 (674), 60 (678), 67 (673) and 69 (669, twins) days old, respectively. Until the age of 67 days the foetuses moved spontaneously. Slight rhythmic movements of respiratory nature were seen, the rhythm of which was a little more frequent than before (foetus of 58 days, 48 per min.). The foetuses of 69 days were quieter; only the one later exposed showed a few small spontaneous movements. Stimulation, e.g. tapping the head or pinching the tail of the foetuses, resulted in somatic movements. Only the twins, especially the first one exposed, showed a more subdued reflex behaviour.

The foetus of 58 days behaved very much like that of 49 days. There was no response to CO_2 and none on tying the cord. Injecting 2μg. of KCN, however, resulted in an immediate deepening of the spontaneous respiratory movements.

The foetuses of 60 and 67 days and the later twin, all responded to CO_2 with rhythmic respiratory movements. Frequencies of 45–78 per min. were observed. Cyanide, 0·24μg. on the foetus of 60 days, and 4μg. on that of 67 days, had an effect similar to that of CO_2. A regular respiratory rhythm started at once. The type of the respiratory movements as well as the rhythm were the same when induced by either of these stimuli.

Anoxia, however, produced an entirely different response. An O_2-poor gas mixture was administered to the mother of the foetus of 60 days. The anoxia did not induce any respiratory rhythm in the foetus, except a single gasp.

Clamping the cord of the foetus of 67 days and of the second twin, similarly, produced a few deep gasps, not nearly as frequent as the rhythm produced by CO_2. Anoxia made the later foetus more sensitive to stimulation.

The first twin differed from the rest of the group by not responding to CO_2. Cyanide was not tried. The somatic reflexes seemed to be under a much stronger inhibition than those of the other twin which was exposed later.

Respiratory movements not induced: movements of non-respiratory nature. The foetuses of 91 (691) and 96 (696) days did not exhibit any spontaneous movements. Administering CO_2 or cyanide did not elicit any respiratory movements. Neither of these, however, was quite without an effect. With CO_2 the foetuses reacted with some movements; rhythmic swallowing, chewing, twitches of ears and of shoulder, spasms not involving the thorax.

Cyanide was given to both foetuses in doses of 4 and 27 μg. Both doses gave a few movements similar to those induced by CO_2. The cyanide did not seriously impair the condition of the foetal C.N.S., for the foetus remained sensitive to the movements of the saline solution.

No movements induced. The rest of the foetuses can all be put under the same heading. This group consists of eleven foetuses whose ages were: 107 (676), 112 (679), 132 (692), 139 (694), 141 (701, 703, 704), 142 (699, 702, twins) and 144 (697) days. The foetuses were quiet, but somatic reflexes could often be elicited, e.g. by pinching the fetlock.

CO_2 was tried on ten of these, cyanide on six. CO_2 never gave any respiratory rhythm, and only occasionally some slight movements were seen.

Cyanide, in the dose of 4 μg., generally had no effect. In the foetuses 692, 694 and 699, 27 μg. induced stretching and a few single movements of respiratory nature, but without a rhythm. In the foetuses 679, 697 and 701, even this dose was without effect. Very much higher doses were needed to give a more substantial reaction. In the foetus of 112 days (679), 13·3 mg. of KCN resulted in gasps and in a respiratory rhythm for a short time. In a foetus of 142 days (699), 2·66 mg. gave small jerks, but in the foetus of 144 days (697) the same dose did not give any somatic movements. Such a dose as 2·66 mg.—it has to be kept in mind—is also of an entirely different order from that effective in the younger foetuses. The foetus of 67 days weighs about 100 g. and at term it may be forty times heavier. 2·66 mg. however, is more than 600 times the dose which proved effective at 67 days.

Chemosensitivity at birth

In order to secure information about the possible role of the chemosensitivity at birth, eight of the foetuses mentioned above were experimented upon, on or after the 139th day. (Term is at 147 days.) In addition to the experiments on CO_2 and cyanide, O_2-poor mixtures were given to three foetuses (702, twins; (704), whose mothers were under spinal anaesthetic. The O_2-poor mixture was administered in each experiment first when the foetus was in the amniotic sac. The anoxia made the foetuses very active, they kicked violently and executed swallowing movements. No distinct respiratory movements were seen, certainly no respiratory rhythms. When, however, the nose of the foetus was kept above the surface of the bath, repeating the same procedure produced in all three foetuses the onset of respiration. It started as gasps, not primarily as a rhythm. Only after tying the cord was a rhythm initiated. Bleeding the lambs produced exactly the same type of behaviour as the O_2-poor mixture; violent kicking, shivering, wriggling and gasping.

When one of the twins had started gasping, still having an intact placental circulation, CO_2 was administered to its mother. It produced no apparent change in the behaviour of the foetus, or lamb. The effect of CO_2 was tested also on the respiration of a lamb later, at the age of 3 days. The response was quite similar to that of an adult.

The important role of external stimuli in comparison to the asphyxial ones was very clearly seen in the following experiment:

Sheep 700, 142 days, spinal anaesthetic (1·5 c.c. duracaine).
Experiment conducted with sheep in saline bath.
The mother is quiet. No hyperventilation.

Caesarean section. The foetus is delivered very quickly after starting the operation. The uterus is opened and the head of the foetus is first taken out, but the amniotic sac is left intact. Immediately the nostrils of the foetus are seen to move rhythmically in a respiratory manner. The foetal body also is very soon seen, and then it shows a rather powerful rhythmical respiration. The condition of the foetus is apparently good, the colour of the umbilical vessels markedly bright. The foetus is quite alive, moves vigorously and has a good tonus. Cord is tied. After opening the amniotic sac the foetus tries to make a sound, not entirely without success, and to move about, but very soon it becomes flaccid and ceases to show any rhythmical breathing. After a considerable period it starts to execute several deep gasps, at irregular intervals. At this time the foetal heart beats rather strongly. A respiratory movement of the nature of a small gasp can always be elicited by tapping the nostril lightly with the finger. When artificial respiration was attempted, much mucous fluid comes from the mouth of the foetus. At the end, the foetus is quite cyanotic, the heart beat weakens, and the foetus obviously dies.

Summarizing the protocol: on opening the uterus and exposing the sac, the foetus, which was obviously in a good condition, was seen to execute rhythmic respiratory movements. Consequently, it inspired amniotic fluid, and after delivery, in spite of artificial respiration, could not get rid of this fluid. An asphyxial onset of the respiration certainly was excluded.

DISCUSSION

How do the results of the present work fit the general picture of the development of the function of the C.N.S. in foetal sheep? 'Spontaneous' rhythmic respiratory movements have been observed from the 40th day onwards. Between the 50th and the 70th day the foetal C.N.S. is gradually becoming inhibited, as the midbrain and, later on, the diencephalic centres begin their function (Barcroft & Barron, 1942; Barron, 1941). In the experiments just described, stimulating effects of CO_2 and of cyanide on the respiratory movements were observed between the 58th and 69th days.

Three possibilities thus come into question. Is this stimulation of respiratory movements by CO_2 and by cyanide: (*a*) a true stimulation, or (*b*) a release of inhibition (as the effect of anoxia is understood), or (*c*) a combination of both?

The last possibility seems to be the most probable one. The nature of the respiratory movements elicited by CO_2 or cyanide was quite different from those resulting from anoxia. The respiratory effect of cyanide was momentary; as the dose used was very small indeed it could not possibly produce a condition of 'general anoxia'. These facts obviously suggest, that we are dealing here with something which has its place under the heading 'stimulation'. On the other hand, the possibility of a second component—the release of inhibition—cannot be entirely ruled out.

No direct attempt was made to determine the localization of the cells or tissues responsible for the stimulation obtained. Any suitable experimental procedure at that age—as far as the authors can see—would mean a rather severe exposure of the foetus, too severe to be stood by a foetus some 65 days old. On the principle of analogy, however, two arguments supporting the reflex nature of the stimulation can be put forward. First, the momentary stimulation of the respiratory movements by cyanide in adult mammals is known to be entirely due to the peripheral chemoreceptors, mainly to those of the carotid body (Comroe, 1939; Verdonk, 1941). The respiratory centre, on the contrary, is depressed by cyanide. In other words, this would mean that the foetus has at the age of 60–70 days a functioning carotid body chemoreflex. And secondly, if it were a reflex it should undergo the same development as all the foetal somatic reflex behaviour; it should become inhibited about the middle of pregnancy and be released at birth. This is precisely what happens.

Does the assumption of a functioning carotid body fit the picture of the anatomically existing structure at this foetal age? In adult sheep the carotid body functions similarly to that of other mammalian species (Van Damme, 1933). Unfortunately, there are no histological data about the carotid body of foetal sheep. Boyd's (1937) work on human foetuses shows, that the microscopical development of the carotid body occurs quite early, mainly between the 12·5 and 36 mm. crown-rump length stages. Watzka's (1937) findings on several other mammalian species give a similar picture. The crown-rump length of the sheep foetus of 58 days was 115 mm.; accordingly, perhaps it may be concluded that the foetus of that age probably has a carotid body.

The carotid body sends its messages to the brain through the glossopharyngeal nerve. This reaches a part of the brain (medulla) which starts its function very early. Hence, the anatomical basis for the onset of the carotid body function probably is there.

In addition to CO_2 and cyanide, the carotid body of adult animals is also sensitive to changes in O_2 pressure. In foetal sheep, anoxia never produced an effect like that of CO_2 or of cyanide. Whether this is due to a difference between foetus and adult at the level of the chemosensitive elements or at that of the correlating or effector mechanisms cannot be judged. Our experiments do not rule out the possibility of an anoxic reflexogenic drive, the effects of which were, however, largely modified by the effect of anoxia on the corresponding centres.

In the youngest foetus which gave a positive response to cyanide, CO_2 was not effective. It might be, that the 'spontaneous' rhythms seen on opening the uterus have been CO_2 rhythms. On the other hand, Rosenfeld & Snyder (1938) in their experiments also found a response to cyanide in some premature rabbits before CO_2 gave any effect.

The third age group, in which CO_2 and cyanide induced general motility but no respiratory rhythms, offers a picture of special interest; there was the rather striking occurrence of rhythmic chewing and swallowing movements, whereas no respiratory rhythms were released. Labial and lingual rhythm, chewing and sucking, all have been understood as a relatively late development of the originally respiratory rhythm (Barcroft, 1941). At this phase, the respiratory 'centre' seems to be under a much stronger inhibition than the closely related centres governing the suctorial movements.

Snyder & Rosenfeld (1937) interpreted their findings on rabbit foetuses and on newborn rabbits as indicating: first, that there is an inhibition of the carotid body reflex mechanism during foetal life; secondly, that the foetal respiratory centre is less sensitive to carbon dioxide than it is after birth. Our observations on sheep between the 70th day of foetal life and birth can be expressed as follows. *There is during the second half of the foetal life an inhibition of the nervous mechanism responsible for the respiratory movements.*

As far as it can be seen, the chemosensitivity at the foetal age of 60–70 days does not serve any immediate purpose. On the other hand, it can teleologically be understood as a manifestation of a phase in the ontogenesis of a function needed in future. It is also worth remembering that a reactivity like this is an old property, present already in aquatic vertebrates; the larvae of toadfish react quite similarly to CO_2 and to cyanide (Tracy, 1926).

The next phase, the development of an inhibition, easily finds its 'usefulness' for the animal. If there were no inhibition of the respiratory reflexes, the result would be that observed in the sheep 700; the foetus would be drowned before it had been born. For a foetus near to term it is as essential not to breathe as it is for a newborn lamb to start breathing! The inhibition is associated with the onset of the function of the regions of the brain anterior to the pons (Barcroft & Barron, 1942). On the mechanism of this inhibition the present experiments do not give much light. The inhibition is something which disappears at birth, so much is certain; but what this 'something' is remains a matter of speculation. It has, perhaps, a counterpart in the behaviour of diving mammals, in which contact of the respiratory openings with water stops breathing (Krogh, 1941). In the experiments on the foetuses older than 140 days taking the nose out of the bath was sufficient to release the gasp mechanism, but to obtain a rhythm it was necessary to tie the cord. On the other hand, the foetus 700 started a respiratory rhythm when in the amniotic sac. This foetus seemed to be strikingly well oxygenated for its age; whether this had anything to do with the too early disappearance of the inhibition, cannot be judged. The inhibition may be due to the combined effect of several factors.

SUMMARY

1. Sheep foetuses between the foetal age of 43 days and term have been subjected to increased CO_2 pressures and intravenous cyanide injections. The effects of these on the foetal respiratory movements have been compared to those of anoxia.

2. The effects of CO_2 and of cyanide can be divided into four groups according to the age of the foetus.

(i) 43–49 days. No effect.

(ii) 58 days. Spontaneous respiratory movements deepened by cyanide; CO_2 without effect.

60–69 days. Both CO_2 and cyanide induced vigorous respiratory movements like the 'spontaneous' respiratory movements.

(iii) 91, 96 days. No induction of respiratory movements; some rhythmic swallowing, chewing, twitching of the ears, etc.

(iv) 107–term. No effect.

3. In 60–69 day foetuses anoxia induced 'gasping' respiratory movements of quite a different character to those induced by CO_2 and by cyanide, and after a much longer latent period.

4. The interpretation of the findings is discussed. The induction of respiratory movements by CO_2 and by cyanide is probably a chemoreflex from the carotid body. The failure to induce such movements in the latter half of pregnancy accords with the fact that somatic reflexes become inhibited at that time, only to be released at birth.

5. Some experiments on the role of chemosensitivity during birth are described. They indicate that cutaneous sensory stimulation rather than asphyxia is responsible for the commencement of breathing.

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AN OUTFLOW RECORDER USEFUL FOR DETECTING SMALL AMOUNTS OF VASOPRESSIN

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The recorder to be described has been used successfully to record the outflow from small perfusions when the flow is between the ranges which can readily be recorded by the drop timer and the outflow recorder of Gaddum (1929, 1938).

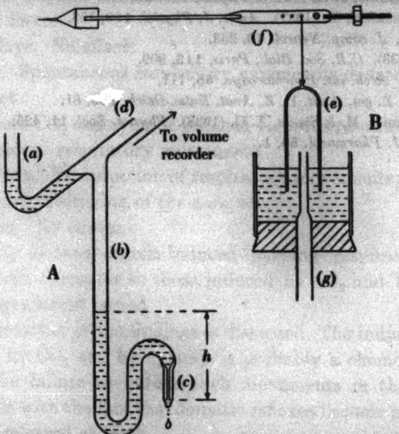

Fig. 1. A, the outflow recorder. The flow enters at (*a*), flows down the tube (*b*) and out at (*c*). The greater the flow the higher the level of fluid in (*b*), air is displaced and the volume recorder B transfers this movement to a smoked drum.

The recorder is shown diagrammatically in Fig. 1 A. The flow to be measured is delivered at (*a*) and runs down the side of the glass tube (*b*) around the S-bend and out at the capillary (*c*).

The rate at which the fluid passes the capillary depends on the pressure due to the height of fluid *h* in the tube (*b*), and for any steady rate of flow the height adjusts itself until the pressure is just sufficient to send this flow out through the capillary. If the flow increases, the level in (*b*) rises until the pressure is sufficient to deal with the new rate. The rise of the level of fluid displaces air and since this is prevented from escaping at (*a*) by the fluid, it is transferred along tube (*d*) to the volume recorder. This volume recorder may be the usual piston recorder, or may be similar to that shown in Fig. 1 B. This consists of a glass dome (*e*) suspended from the arm of a writing lever (*f*) over the opening of a tube (*g*) in which the displaced air is moved. The glass dome and the tube (*g*) are both coated with a thin layer of paraffin wax in order to prevent the surface tension of the surrounding water drawing them together.

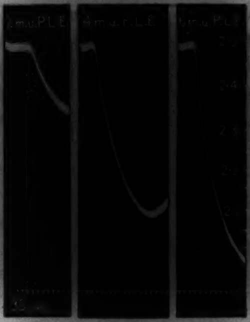

Fig. 2.

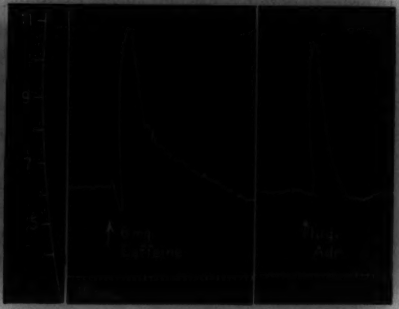

Fig. 3.

Fig. 2. Record of the outflow from the hind legs of a rat perfused with Ringer-Locke; approximate scale in ml. per min. Constrictions produced by 2, 4 and 6 m.u. of posterior lobe extract.

Fig. 3. Record of the coronary flow of a rabbit heart (Langendorff preparation) with approximate scale in ml. per min. Dilatations produced by 6 mg. caffeine sodium salicylate and 1 μg. adrenaline.

The sensitiveness of the recorder depends on several factors: (1) the resistance of the capillary tube which depends on its length and diameter. The greater the resistance the greater is the volume change produced by a change of flow. (2) The diameter of the vertical tube (*b*); an increase in its diameter necessitates a larger volume change in order to produce a given pressure change. In addition the sensitivity of the whole apparatus depends on that of the volume recorder. Thus the recorder may be made sensitive to the small changes in flow either by having a very sensitive volume recorder or by producing large volume changes from the changes in flow. A large volume change, however, is only obtained at the expense of an increased delay before the new level is attained.

As it has been used the dimensions of the apparatus have been as follows: length of writing lever 31 cm., distance of attachment of glass dome from fulcrum 1·5 cm., the diameter of the dome 1·5 cm. For the tracing shown in Fig. 2 the diameter of tube (*b*) was 0·45 cm. and the capillary approx. 1 cm. long and 0·07 cm. in diameter. For Fig. 3 the dimensions were 0·7, 1 and 0·035 cm.

Detection of vasopressin. The blood vessels of the rat are very sensitive to the action of the extract from the posterior lobe of the pituitary gland, and provide a sensitive test for detecting the presence of the pressor hormone. When a preparation of the hind legs of a rat, perfused through the abdominal aorta with Ringer-Locke at room temperature, has been working for 4–5 hr. it will respond to 2 m.u., some preparations responding well to 1 m.u. or less. In order to obtain consistent effects 15–20 min. must elapse between injections, but the life of the preparation is long, and responses can still be obtained when the preparation is 24 hr. old. Fig. 2 shows the effect of 2, 4 and 6 m.u. The oscillations of the record are due to the individual drops of perfusate arriving in the tube, each drop causing a sudden increase in the level of fluid, which then declines again until the arrival of the next drop.

Record of coronary flow. The apparatus has also been used for recording the outflow from an isolated heart perfused through the coronary vessels by Langendorff's method. Fig. 3 records the outflow from an isolated rabbit's heart; the dilatations are produced by 6 mg. of caffeine and 1 μg. of adrenaline. In many rabbit hearts adrenaline produces little coronary dilatation, but this is not always so.

SUMMARY

An outflow recorder is described which gives a continuous record. The height of the line is an indication of the flow. The instrument is especially useful for recording the flow from the perfused vessels of the rat, which are sensitive to amounts of pituitary posterior lobe extract equal to 1 m.u. or less.

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THE LOCAL ELECTRIC CHANGES ASSOCIATED WITH REPETITIVE ACTION IN A NON-MEDULLATED AXON

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One of the most striking properties of crustacean nerves is that they readily give rise to repetitive discharges when stimulated with weak constant currents. Fessard (1936) and Arvanitaki (1938) have made extensive studies of the repetitive discharges which can be recorded in multifibre preparations from *Carcinus maenas* and *Cancer pagurus*. The present experiments deal with the behaviour of isolated axons from *Carcinus maenas*. The results obtained are in general agreement with those of Fessard and Arvanitaki, but give more precise information about the local electric changes associated with repetitive action than can be obtained from studies of whole nerve trunks. The repetitive discharges of crustacean axons are of general interest for two reasons. In the first place the characteristics of the discharge are often very similar to those recorded from sensory receptors or motor neurones. Great caution must be exercised in applying conclusions about invertebrate nerve to the excitable cells of vertebrates. But vertebrate receptors and motor neurones resemble crustacean nerve in at least one important respect. According to Cajal (1899) most sense organs or motor neurones contain a short stretch of non-medullated axon, and this may behave in a manner that is more closely simulated by the thinly myelinated axons of crustacea than by the heavily myelinated nerve fibres of vertebrates. The repetitive discharges of crustacean nerve are also important because they raise an interesting physical question. It will be shown that an axon with a spike duration of 1 msec. and a relative refractory period of less than 10 msec. may repeat at frequencies as low as 5/sec. The frequency of such a discharge must be regulated by a process which has slower time relations than the spike or the recovery cycle and the experiments of Arvanitaki (1936) indicate that this process has an electrical sign. In her more recent work Arvanitaki (1939) has shown that the repetitive discharges of decalcified *Sepia* nerve are associated with the oscillations of a local electric process. A similar conclusion may be drawn from the work of Adrian & Gelfan (1933) on decalcified frog's muscle or from that of Brink, Bronk & Larrabee (1946) on the decalcified nerves of frog and squid. The subthreshold potentials of *Carcinus* axons show little tendency to oscillate provided that weak currents are employed and that the axons are kept in solutions of normal ionic content. But axons repeat without these special conditions and it is with the local electric changes which accompany such repetition that this paper is concerned.

METHOD

The apparatus employed did not differ materially from that described in former papers (Hodgkin, 1938; Hodgkin & Rushton, 1946; Hodgkin, 1947*a*). A single fibre was mounted on three silver chloride electrodes which terminated in fine agar wicks. Current was applied to the central electrode and to one of the adjacent electrodes through a resistance of 10–300 MΩ. Electrical changes were recorded from the central electrode and the remaining electrode with a d.c. amplifier of high input impedance. The central electrode was earthed and the usual precautions to avoid leaks or artefacts were employed. Electrical changes recorded in this way may be taken as being roughly proportional to those at the point where impulses arise. The absolute magnitude of the membrane potentials must have been reduced to about one-half by the short-circuiting effect of the external fluid (Hodgkin, 1947*a*; Hodgkin & Rushton, 1946) but no distortion of time course was introduced by this cause. The recorded changes were slightly distorted by the potential drop resulting from the flow of applied current in the common stimulating and recording electrode. With the type of electrode employed the magnitude of this effect normally amounted to about 3 % of the subthreshold potentials and to less than 1 % of the spike. It could therefore be ignored with safety. Another source of error arose from the finite width of the central electrode. The potential difference across the nerve membrane was recorded from the extrapolar edge of the central electrode whereas impulses probably arose at the interpolar edge. The difference between the membrane potentials at the two sides of the central electrode probably amounted to about 6 % since the electrode width was of the order of 200 μ. and the appropriate space constant for these axons was about 2·5 mm.

The stimulating current consisted either of an electronically generated rectangular pulse or of a constant current applied by means of a morse key. Determinations of the refractory period and recovery curves were made with an electronic circuit which generated two short pulses of variable spacing and amplitude.

Axons were normally kept in sea water before being raised into paraffin oil. A few preliminary experiments were made with *Carcinus* Ringer (Hodgkin, 1947*b*) but did not reveal any obvious difference in the pattern of repetitive behaviour.

The experiments were made at temperatures between 14 and 16° C.

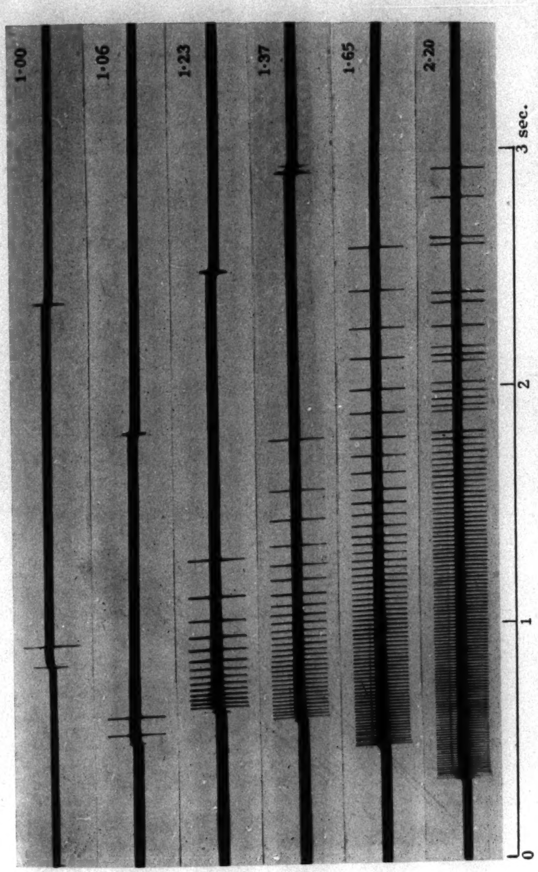
RESULTS

The characteristics of the response to a constant current varied considerably in different axons (cf. Fessard, 1936; Arvanitaki, 1938). The preparations encountered could be divided into three main groups:

(1) Axons in which the recovery cycle showed no significant supernormal phase and which were capable of repetition over a wide range of frequencies. In such axons the frequency varied smoothly over a range of about 5–150 impulses per sec.

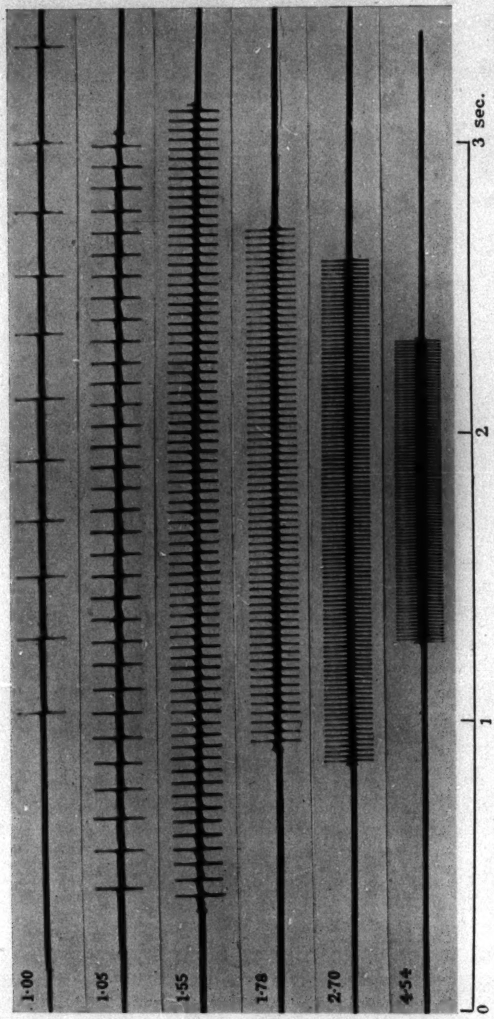
(2) Axons with a pronounced supernormal phase. This class usually gave a train of impulses of frequency 75–150/sec. which was relatively insensitive to changes in the strength of the applied current.

THE JOURNAL OF PHYSIOLOGY, Vol. 107, No. 2 PLATE 1



Electrical changes at stimulating electrode produced by sudden application of constant currents. The numbers give the current strength relative to the rheobase. Transient artefacts are visible at make and break.

To face p. 166



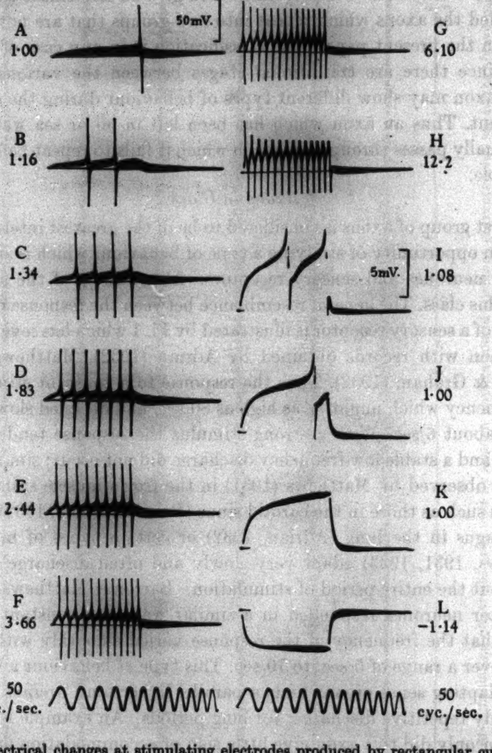
Electrical changes at stimulating electrode produced by sudden application of constant currents with strengths shown relative to rheobase. The absolute magnitude of the rheobase was about $3 \cdot 5 \times 10^{-9}$ amp. The beginning and end of the applied current is marked by a slight artefact.

(3) Axons with high threshold and low safety factor which either failed to repeat or succeeded only if the current strength was much greater than rheobase. The response time of these axons never reached such large values as that of axons in the first two classes.

This classification was based on the present experiments, but is similar to that adopted by Arvanitaki (1938). Arvanitaki recognized a class of axons which repeat only with difficulty and which give an immediate response, while she divided the axons which repeat into two groups that are not unlike those defined in the present paper. The classification is in any case of an arbitrary nature since there are transitional stages between the various groups and a single axon may show different types of behaviour during the course of one experiment. Thus an axon which has been left in oil or sea water for many hours usually passes through a stage in which it fails to repeat before becoming inexcitable.

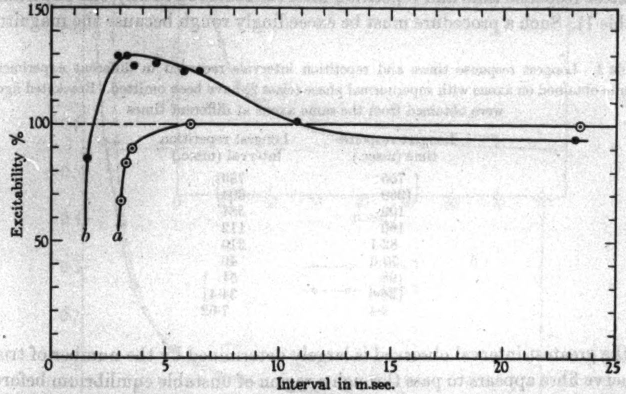
Axons in Class I

The first group of axons is considered to be of the greatest interest because it affords an opportunity of studying a type of behaviour which is similar to that of motor neurones and sensory receptors. About 50 % of the axons studied were in this class. The general resemblance between the response of a crab fibre and that of a sensory receptor is illustrated by Pl. 1 which has several properties in common with records obtained by Adrian (1932), Matthews (1931) and Hartline & Graham (1932). Thus the response to a constant stimulus started at a frequency which might be as high as 80/sec. and declined slowly to a lower limit of about 6/sec. With a strong stimulus the response tended to become irregular and a stable low frequency discharge did not occur; similar behaviour has been observed by Matthews (1931) in the frog's muscle spindle. Sensory receptors such as those in the carotid sinus (Bronk & Stella, 1932), the endings of the vagus in the lung (Adrian, 1932) or certain types of muscle spindle (Matthews, 1931, 1933) adapt very slowly and often discharge continuously throughout the entire period of stimulation. Barron & Matthews (1938) found that motor neurones responded in a similar way to a constant current and showed that the frequency of the response varied smoothly with the applied current over a range of 5/sec. to 70/sec. This type of behaviour and that of the slowly adapting sense organs finds a parallel in certain *Carcinus* axons which give steady repetitive discharges for long periods. An example is provided by Pl. 2. Not only did this axon give little sign of accommodation or fatigue, but the frequency of the discharge showed an initial increase with time. The increase in frequency must have been due to some progressive rise in excitability or 'warming-up' process, but no attempt was made to discover the nature of the progressive change which was observed on infrequent occasions. The repetitive train seen in the uppermost record of Pl. 2 ended with the last impulse shown. In the remaining records the repetitive response was terminated only by the removal of the stimulating current. Records taken earlier from this axon indicated that the repetitive discharge produced by a 1·4 rheobasic current was maintained at a frequency of about 16/sec. until the end of a stimulus lasting 17/sec. The stimulus was not continued for a longer period because it was feared that prolonged activity might cause an irreversible change in the characteristics of the axon.



Text-fig. 1. Electrical changes at stimulating electrodes produced by rectangular currents with strengths shown relative to rheobase. The voltage calibration in A applies to records A–H and that in I to records I–L. The rheobase in I–L was 22 % higher than that in A–H. The absolute magnitude of the current in A was about 8×10^{-9} amp.

Details of the response to a rectangular current are given in Text-fig. 1 which illustrates a number of important points. In the first place it can be seen that the local subthreshold potentials were small compared to the propagated action potential. Thus the depolarization produced by a just subthreshold current (record K) amounted to about 6·2 mV. whereas the spike amplitude was 57 mV. Another point of interest is that the response time was extremely long when the strength of current was weak. The delay in A amounted to 98 msec. and it is probable that longer delays would have been observed if the pulse could have been increased in duration. Further, the interval between the beginning of the current and the first spike in records B–F was of the same order as the interval between impulses in the repetitive train. This observation suggests that response time rather than refractory period is the primary factor in determining the frequency of repetition when the current is weak. Fessard (1936) and Arvanitaki (1938) came to a similar conclusion in their studies of whole nerve trunks from *Carcinus maenas* and other crustacea. The hypothesis receives support from a number of directions. The relative refractory period has been found to occupy less than 10 msec. in an axon which was capable of repetition at 5/sec. An example is afforded by Text-fig. 2*a* which gives the recovery curve of the axon used in obtaining the records in Pl. 2.



Text-fig. 2. Curve *a*. Recovery curve of an axon in class 1. Curve *b*. Recovery curve of an axon in class 2. Abscissa. Interval between two shocks in msec. Ordinate (normal threshold)/(threshold during recovery period).

The recovery curve was not determined with great precision and it might be argued that recovery from refractoriness was not quite complete at the end of 10 msec. and that it remained incomplete for 200 msec. This argument is not a plausible one and it breaks down completely when the effect of currents appreciably greater than rheobase is considered. If recovery from refractoriness were the sole factor in determining repetition rate the axon of Text-fig. 2*a* and

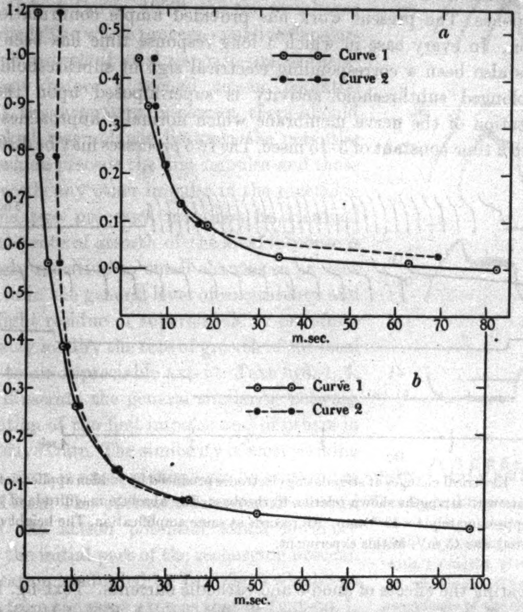
Pl. 2 should discharge at 240/sec. when the current was 5 % above rheobase. The actual repetition rate was about 13/sec. at this strength. Persistent changes in excitability such as the subnormal period and the second period of supernormality described by Gasser (1937) may have a considerable effect in modifying the rhythm set by the rate of growth of the local response, but they do not appear to be the most important factor in crustacean axons.

In order to explain the low repetition frequencies in terms of response time it would be necessary for this time to be of the same order of magnitude as the repetition interval. A rough comparison between the two intervals was made by examining a large number of photographic records and determining the greatest response time and repetition interval observed in any one experiment (Table 1). Such a procedure must be exceedingly rough because the magnitude of the greatest interval observed is largely determined by the number of trials. A nerve fibre appears to pass through a region of unstable equilibrium before it propagates and the length of time for which it can remain in this condition is plainly incapable of precise definition. But Table 1 indicates that the maximum response time is usually of the right order of magnitude to account for the longest repetition interval observed in any particular experiment. There is also an obvious correlation between the two sets of figures and the correlation coefficient between the logarithms of the two quantities was found to be 0·88 which can be shown to be a highly significant result.

TABLE 1. Longest response times and repetition intervals recorded in different experiments. Results obtained on axons with supernormal phase (class 2) have been omitted. Bracketed figures were obtained from the same axons at different times

Longest response time (msec.)	Longest repetition interval (msec.)
755	789
1980	604
109	584
160	112
82·1	210
70·6	49
98	51
24·6	34·4
4·4	7·62

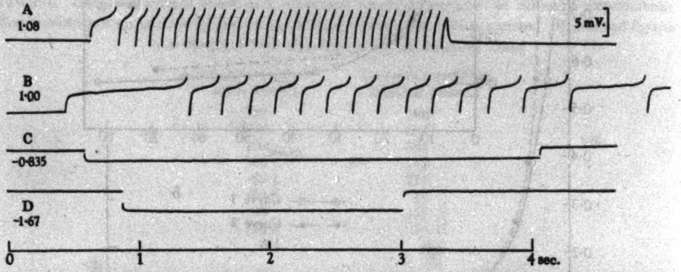
A better method of comparing response time with repetition interval is to plot these quantities against the strength of current. The relation between response time and current strength is illustrated by curve 1 of Text-fig. 3*a* and *b*, while curve 2 shows the effect of current on the initial repetition interval. The two intervals were usually not identical and showed considerable deviations near rheobase, but both were affected by current in a comparable manner and the general similarity between the two curves suggests that both were determined by a common process. The refractory period became important when the current strength was large and the repetition frequency was high. Strong currents reduced the response time towards zero but the repetition interval never became less than 6·5 msec. and in one case actually increased when the current was raised sufficiently. An explanation of this phenomenon may be that recovery from refractoriness is hindered by strong cathodal depolarization (cf. Bishop & Erlanger (1926)). The decrease in action potential height which



Text-fig. 3*a* and *b*. Effect of current strength on response time and repetition interval in two axons. Curve 1, interval between make of current and appearance of first impulse; curve 2, interval between 1st and 2nd impulses. Abscissa: interval in msec. Ordinate $\log_{10}$ (current/rheobase).

can be seen in Text-fig. 1 is probably another sign of the depressant action of excessive depolarization. The resemblance between the two curves in Text-fig. 3*a* and *b* would have been less if the process of accommodation had been more rapid. Arvanitaki (1938, p. 52) presents experimental data of this kind but shows that the entire family of curves relating current strength to the intervals which precede the 1st, 2nd, 3rd... spikes can be superposed if the current strength is expressed in terms of the individual rheobases of the 1st, 2nd, 3rd... spikes. The excellent fit which she obtaines provides strong evidence for the hypothesis that the evolution of the first impulse is essentially similar to that of any other impulse in the repetitive train.

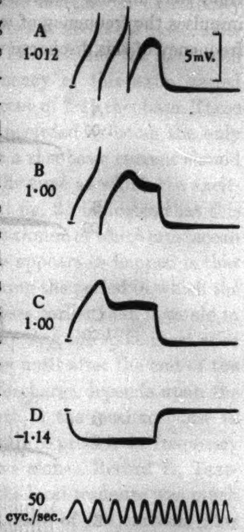
The preceding arguments indicate that the large response times observed in *Carcinus* axons are associated with the ability of these axons to give stable low frequency discharges. Hodgkin & Rushton (1946) reported that the long response times of *Carcinus* axons were themselves associated with a prolonged local response. The present work has provided ample confirmation of this conclusion. In every case in which a long response time has been observed there has also been a corresponding electrical sign of subthreshold activity. This prolonged subthreshold activity is superimposed upon the passive depolarization of the nerve membrane which normally approaches a steady state with a time constant of 5–15 msec. The two processes may be distinguished by comparing the effects of anodic and cathodic currents. Text-fig. 1 record L gives the effect of a weak anodic current while records K and J give the effects of just subthreshold and just threshold currents. The difference between the two curves is most easily explained by postulating a subthreshold process which grows at a rate depending on the applied current until it reaches propagating size.



Text-fig. 4. Electrical changes at stimulating electrodes produced by sudden application of constant currents with strengths shown relative to rheobase. The absolute magnitude of the rheobase was approximately 4×10^{-9} amp. All records at same amplification. The height of the action potential was 45 mV. in this experiment.

The rate of growth of the local response may be exceedingly slow if the current strength is only just sufficient to excite. An extreme example is afforded by Text-fig. 4. In this case the local activity developed in a slightly irregular manner but, nevertheless, showed an upward trend during 950 msec. On the other hand, the passive charging process was complete in about 20 msec. as may be seen from records C and D.

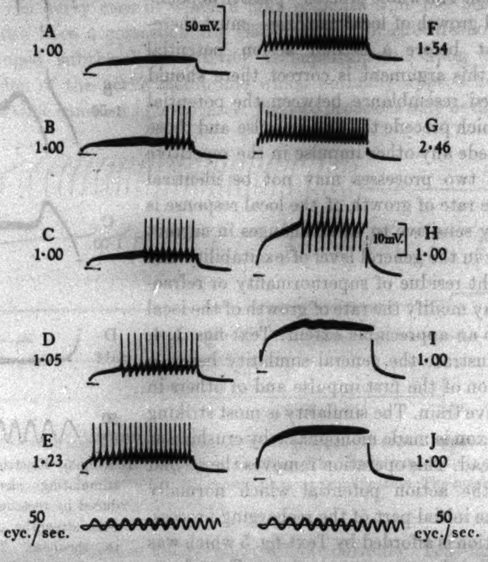
The sequence of events in a repetitive discharge may be summarized in the following way. When current is suddenly applied, the membrane charges to a value determined by the membrane resistance with a time constant of 5–15 msec. Upon this charging process is superimposed the slow growth of the local response. The effect of both processes is to depolarize the axon and a propagated action potential arises when a critical level of depolarization is reached (Hodgkin & Rushton, 1946, fig. 15). The membrane resistance falls to a low value during activity (Cole & Curtis, 1939) and the membrane is discharged. The whole process—passive depolarization and growth of local response—must therefore repeat before a second action potential arises. If this argument is correct there should be a marked resemblance between the potential changes which precede the first impulse and those which precede any other impulse in the repetitive train. The two processes may not be identical because the rate of growth of the local response is exceedingly sensitive to small changes in current strength or in the general level of excitability and a very slight residue of supernormality or refractoriness may modify the rate of growth of the local response to an appreciable extent. Text-figs. 1, 4, 5 and 7 illustrate the general similarity between the initiation of the first impulse and of others in the repetitive train. The similarity is most striking when the axon is made monophasic by crushing at the distal lead. This operation removes the second phase of the action potential which normally obscures the initial part of the recharging process. An illustration is afforded by Text-fig. 5 which was obtained from the same axon as that in Text-fig. 1. Unfortunately the operation of crushing the axon caused some alteration in the characteristics of this axon. The response time at rheobase became shorter and the local response rose to a definite maximum during the rectangular pulse. These changes were probably caused by the spread of depolarization from the crushed point as a result of local circuit action. But the records show plainly that the electrical changes which preceded the first impulse were very similar to those which preceded the second.



Text-fig. 5. Electrical changes at stimulating electrodes produced by rectangular currents with strengths given relative to rheobase. The absolute magnitude of the rheobase was about 8×10^{-9} amp. All records at same amplification.

Axons with pronounced supernormal phase (*Class* 2)

Text-fig. 6 is an example of the response of an axon with a pronounced supernormal phase. The initial part of the response was not markedly different from that already described, but the first spike was followed by a train of impulses the frequency of which bore little relation to the response time. The frequency of this discharge was relatively insensitive to changes in the applied current. In the experiment illustrated by Pl. 2 the frequency was trebled where the current strength was increased from rheobase to 1·05 rheobase; in the experiment of Text-fig. 6 it rose by only 19 % for an increase from rheobase to 1·23 rheobase. A further point is that it was impossible to evoke a single impulse with a current of long duration. At a constant strength of current the axon gave either a train of impulses of fairly high frequencies or no propagated action potentials at all. Axons of this type were not capable of giving low frequency discharges. When the current was maintained for a long time it evoked a train of impulses which might last for several seconds but which ended



Text-fig. 6. Electrical changes at stimulating electrode produced by sudden application of rectangular currents with strengths given relative to rheobase. The voltage calibration in A applies to records A–G and that in H to records H–J. The rheobase in H–J was 2 % higher than that in A–G. The absolute magnitude of the rheobase was about 6×10^{-9} amp.

abruptly without giving frequencies lower than about 50/sec. The obvious explanation of this type of behaviour is that each action potential was followed by a supernormal phase which caused another action potential to appear with relatively little delay. Text-fig. 2*b* gives the recovery curve of the axon which has just been considered. It can be seen that the increase in excitability amounted to nearly 30 % and that it was a maximum at about 3·5 msec. after the first stimulus. Supernormality ended at about 11 msec. and the axon then became slightly subnormal. The repetition frequency of this axon varied between 90/sec. at rheobase and 150/sec. for a current of 2·41 rheobase. These figures are unintelligible if the recovery curve is regarded as being the only factor in determining repetition rate. In that case a rheobasic current should give a frequency which would be determined by the time at which the excitability first returned to normal. The curve of Text-fig. 2*b* indicates that this frequency would be 540/sec., which is close to the maximum of which crustacean axons are capable (Hodgkin, 1938). What actually appears to happen is that the local response develops at an increased rate during the period in which the axon is supernormal. This means that the spike arises earlier than it would in the absence of supernormality, but it does not imply that the spike must arise at the crest of the recovery curve, nor need it arise until after the end of the supernormal phase. The timing of the repetitive discharge depends upon the form of the recovery curve and the rate of growth of the local response in a complicated manner and it would be quite impossible to predict the frequency of the repetitive discharge from the recovery curve alone. Record H, Text-fig. 6 leaves little doubt that the development of the local response was much faster after the first spike than it was after the make of the current. This conclusion is supported by other records and by the previous observation (Hodgkin, 1938) that a subthreshold shock of constant strength produced a larger local response in supernormal nerve than it did in normal nerve.

An interesting feature of Text-fig. 6 is the presence in record I of small subthreshold oscillations of frequency 44/sec. The amplitude of these oscillations varied from stimulus to stimulus; extreme limits are shown by records I and J. It is difficult to make any reliable statement about the nature of these oscillations since they have only been observed on a few occasions. But they appear to differ in several respects from the oscillations seen in decalcified nerves or in nerves stimulated with strong currents. In the first place the oscillations seem to occur over a very narrow range of currents and the variation in amplitude suggests that they only occur when the axon just fails to propagate. A further point is that both the amplitude and the frequency of the oscillations are considerably less than those which can occur in decalcified nerve.

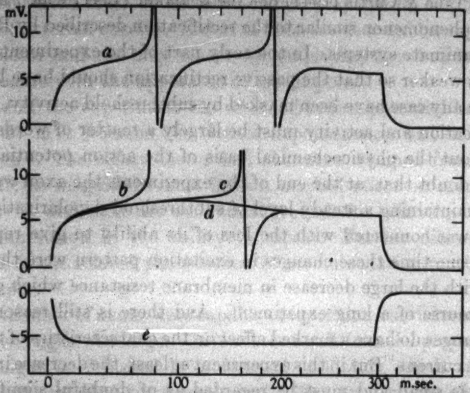
Axons which repeat only with difficulty or not at all (Class 3)

Carcinus axons usually give repetitive discharges if reasonable care has been taken with the dissection. The ability to repeat diminishes if the axons are left in sea water for a long period. Under these circumstances the pattern of the subthreshold potentials alters in a characteristic manner. The response time at rheobase is greatly reduced and the local response rises quickly to a well-defined maximum.

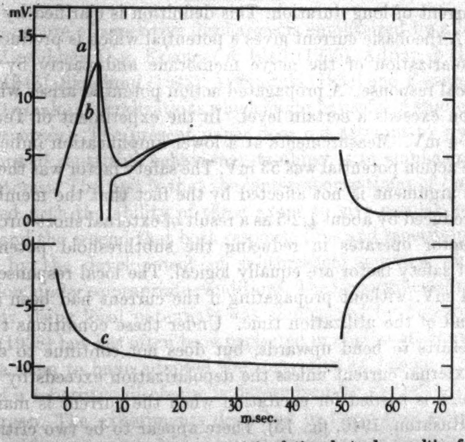
Table 2. Properties of an axon at different stages of an experiment. The left-hand column gives values obtained at about 3 hr. after isolation of the axon and the right-hand column gives values at about 6 hr. after isolation

Magnitude of action potential (external recording)	52·9 mV.	36·6 mV.
Critical level of depolarization (external recording—see text for definition)	7·4 mV.	12·9 mV.
Safety factor (see text)	7·15	2·84
Membrane resistance	8200 Ω cm.^{2}	7200 Ω cm.^{2}
Membrane time constant	8·2 msec.	7·0 msec.
Membrane capacity	1·0 μF. cm.$^{-2}$	0·97 μF. cm.$^{-2}$
Current through axon at rheobase	$9{\cdot}3 \times 10^{-9}$ amp.	$24{\cdot}8 \times 10^{-9}$ amp.
Longest response time observed at rheobase	160 msec.	5·9 msec.
Time to crest of local response (extreme recorded)	120 msec.	7·1 msec.
Lowest repetition frequency observed	9/sec.	—
Number of impulses produced by current equal to twice the rheobase	70	1
Initial frequency produced by 2 × rheobasic current	89/sec.	—

The changes in the excitation pattern of one axon are illustrated by Text-figs. 7 and 8 while Table 2 summarizes the results of a number of quantitative measurements on the same axon. The records in Text-fig. 7 were obtained about 3 hr. after the process of dissection had been completed. The behaviour of the axon at this stage clearly falls into the first class. The response time at rheobase amounted to 160 msec. and the subrheobasic local response rose to a rather flat crest in about 120 msec. The subthreshold response appeared to be maintained throughout the entire period of current flow if the difference between cathodic and anodic curves is taken as a criterion of activity. The repetition frequency varied between 9/sec. at 1·0 rheobase and 89/sec. at twice rheobase; while a twice rheobasic current of very long duration gave rise to about 70 impulses. The records in Text-fig. 8 were obtained about 3 hr. later and show that a marked change had taken place in the characteristics of the axon. The response time did not exceed 6 msec. and the local response rose to a sharp maximum in 7 msec. The axon did not repeat at any strength of current up to 2·5 rheobase and the rheobase itself had increased considerably. The steady potential produced by a rheobasic cathodic current was smaller than that produced by the corresponding anodic current whereas it had formerly been greater. This effect can be attributed to a rectification of the type



Text-fig. 7. Traced records of potential changes at stimulating electrode resulting from rectangular currents with strengths: *a*, 9·5 × 10^{-9} amp. cathodic; *b*, *c* and *d*, 9·3 × 10^{-9}. amp. cathodic; *e*, 10·5 × 10^{-9} amp. anodic. The later part of record *b* has been omitted; it was similar to the corresponding part of record *c*.



Text-fig. 8. Traced records of potential changes at stimulating electrodes resulting from rectangular currents with strengths: *a*, 25·0 × 10^{-9} amp. cathodic; *b*, 24·6 × 10^{-9} amp. cathodic; *c*, 28·0 × 10^{-9} amp. anodic. The records were obtained from the same axon as those in Text-fig. 7 but 3 hr. later.

described by Cole & Curtis (1941) or Cole & Baker (1941) which may perhaps be a passive phenomenon similar to the rectification described by Blinks (1930) in certain inanimate systems. In the early part of the experiment the current strength was weaker so that the passive rectification should have been smaller and would in any case have been masked by subthreshold activity. Arguments about rectification and activity must be largely a matter of words until more is known about the physicochemical basis of the action potential, but there can be little doubt that, at the end of the experiment, the axon was no longer capable of maintaining a steady level of subthreshold depolarization and that this change was connected with the loss of its ability to give repetitive discharges. At one time these changes in excitation pattern were thought to be associated with the large decrease in membrane resistance which often occurs during the course of a long experiment. And there is still reason to believe that such changes do have a marked effect on the characteristics of the response to a constant current. But in this experiment at least, the decrease in membrane resistance was small and must be regarded as of doubtful significance. The most important change which took place during the course of the experiment was the large change in safety factor (Table 1). The concept of a safety factor was introduced to describe the effectiveness of the action potential as an electrical stimulus (Hodgkin, 1937). The safety factor can be defined quantitatively in several ways. In Table 2 it is taken as the ratio of the peak of the action potential to the maximum depolarization produced by a just subthreshold current of long duration. This definition is clarified by reference to Text-fig. 7. A rheobasic current gives a potential which is produced partly by passive depolarization of the nerve membrane and partly by an actively generated local response. A propagated action potential arises when the total depolarization exceeds a certain level. In the experiment of Text-fig. 7 this was about 7·4 mV. Measurements at a lower amplification indicate that the height of the action potential was 53 mV. The safety factor was therefore taken as 7·15. The argument is not affected by the fact that the membrane action potential is reduced by about 1/2·5 as a result of external short-circuiting, since the same factor operates in reducing the subthreshold potential. Other definitions of safety factor are equally logical. The local response could have exceeded 7·4 mV. without propagating if the current had been switched off before the end of the utilization time. Under these conditions the potential time curve starts to bend upwards, but does not continue to do so in the absence of external current unless the depolarization exceeds by about 50 % the level which is needed for excitation when the current is maintained (cf. Hodgkin & Rushton, 1946, fig. 15). There appear to be two critical levels of depolarization in a nerve fibre. The first defines the maximum depolarization which can occur without giving rise to a propagated impulse when the external current is maintained at a constant value. The second defines the level of depolarization which must be exceeded before propagation can take place in the absence of external current. Either of these levels could be used in defining the safety factor and the only reason for employing the first is that it was easier to measure with the apparatus at my disposal.

At the end of the experiment the recorded action potential had decreased from 53 to 37 mV. whereas the critical depolarization had increased from 7·4 to 12·9 mV. The safety factor had therefore declined from 7·4 to 2·8. A change of this magnitude must have a marked effect on many phases of nervous activity and there are theoretical grounds for believing that it would lead to a diminution in the ability to repeat. Further discussion of this problem and of the related phenomenon of accommodation must be deferred to a later paper.

A certain number of axons were capable of repetition but did not do so unless the current strength was large. Under these conditions the propagated action potentials might be followed by a damped oscillation which appears to be similar to that described by Arvanitaki (1939). An example of this type of behaviour was given in a former paper (Hodgkin & Rushton, 1947, fig. 14) and requires no further description. Oscillations are also seen in decalcified nerve, but are not easy to observe since removal of calcium often leads to a prolonged and uninterrupted discharge of nervous impulses.

CONCLUSION

The variable nature of repetitive discharges is emphasized by a large body of experimental evidence [e.g. Erlanger & Blair (1935), Adrian & Gelfan (1933), Brink *et al.* (1946), Skoglund (1942), Arvanitaki (1938) and Fessard (1936)] and it is difficult to make generalizations which might be useful in studies of neurones or sensory receptors. The present paper does not attempt to give a comprehensive theory of repetitive behaviour: its object is to emphasize two points of general interest. The first is that the long response times of *Carcinus* axons are associated with the ability of these axons to give regular low frequency discharges; and the second that both response time and repetition interval are determined by the development of subthreshold activity which may be extremely slow under appropriate conditions. The importance of response time and of slowly rising local potentials was emphasized by Fessard (1936) and Arvanitaki (1938) but has often been neglected in studies of sensory receptors. Most writers seem to have adopted the suggestion of Adrian (1928) that the frequency of a repetitive discharge is determined by the refractory period of the sense organ. The experiments described in this paper suggest that the response time of a sense organ to a constant stimulus may be quite as important as its refractory period.

SUMMARY

The initiation of repetitive discharges by constant currents was investigated by recording local electric changes from the stimulating electrode in isolated axons from *Carcinus maenas*.

The following classification was made on the basis of the types of repetition encountered:

(1) Axons which were capable of responding over a wide range of frequencies (5–150/sec.).

(2) Axons with a pronounced supernormal phase. This class gave a train of impulses of frequency 75–150/sec. which was relatively insensitive to the strength of the applied current.

(3) Axons with high threshold and low safety factor which either failed to repeat or succeeded only if the current strength was much greater than rheobase.

Axons which were left in oil or sea water for long periods usually passed through a stage in which they failed to repeat before becoming inexcitable.

The repetitive behaviour of axons in the first class is of interest because it is similar to that of slowly adapting sensory receptors or motor neurones. The frequency of repetition appears to be primarily determined by the rate of growth of a local electric response to the level necessary for propagation. Thus there is a marked resemblance between the potential changes which precede the first impulse and those which precede any other impulse in the repetitive train. The response time of the first impulse is of the same order as the initial repetition interval and both periods are affected by current strength in a similar manner. The rate of growth of the local response may be exceedingly slow and both response time and repetition interval have exceeded 500 msec. on certain occasions.

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PHOSPHATE CLEARANCES IN INFANTS AND ADULTS

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Phosphate clearances in man have received comparatively little attention but the rate of excretion of P has been studied from time to time. Kleitman (1925) found that the excretion of P rose during sleep and was at its lowest during the forenoon. This was a confirmation of many previous observations (Fiske, 1921). Ollayos & Winkler (1943) investigated the excretion of P in relation to its serum level. Subjects were examined when they were asleep, at rest, fasting and after a meal. The authors found that the rate of excretion of P did not depend upon the level of P in the serum unless the latter had been raised by phosphate infusions. From their paper it appears that when the serum contained 3–4 mg. of inorganic P/100 c.c. the excretion rate varied widely and for unknown reasons from 0·1 to about 1·0 mg./min. This range would have given clearances for normal people of the order of 15 c.c./min. The data given by Blatherwick, Bell & Hill (1924) make it possible to calculate the phosphate clearances of their normal subjects, and they seem to have ranged from 5·8 to 13·3 c.c./min. and to have averaged about 8 c.c./min. This seems a low figure but the experiments were made on fasting subjects and probably during the forenoon when the phosphate clearances were likely to have been lower than at other times of the day or night (Fiske, 1921; Kleitman, 1925, and see below). According to Pitts & Alexander (1944) there is a sharp limit to the rate at which the tubules can reabsorb phosphates. This Tm for phosphates can be depressed by raising the plasma glucose but it is not affected by phloridzin. No work appears to have been done on babies.

METHODS

Urines were obtained from healthy British and German men and women. The times over which urine had been allowed to collect in the bladder were carefully noted. Specimens were obtained at any convenient time; some were passed on rising and others during the forenoon, afternoon or evening. Several specimens were obtained from most of the subjects, rarely only a single one. Blood was withdrawn from an anticubital vein and centrifuged quickly without the addition of any anticoagulant. So far as possible the blood was taken in the middle of the time over which urine was being collected, and in a few instances a second sample of blood was withdrawn.

The 'newborn' infants used for this investigation were mongols or suffered from inoperable meningomyeloceles. The older child had an advanced degree of hydrocephalus. Urine was collected by catheter; blood was withdrawn from the anterior fontanelle in almost every case, and rapidly separated without the addition of any anticoagulant. The exact technique of this work has been described by Dean & McCance (1947). The longer experiments on babies 1 to 4 lasted 6 or 8 hr., and during this time no food was administered by mouth. The tests on the other infants were made between one feeding time and the next, and never lasted more than 3 or 4 hr. Some of the babies were tested on two separate occasions, others only once, and the number of samples of urine and blood which were taken also varied from child to child. The infants appeared to suffer no inconvenience or harm as the result of these studies.

The inorganic phosphate in the serum was determined by Fiske & Subbarow's (1925) method, and in the urine by the same method, or by that of Briggs (1922).

RESULTS

Tables 1 and 2 show the results which have been obtained in adults and children respectively. The clearances shown in the tables are averages of the clearances found in the separate experimental periods. The clearances in adults varied from 8·9 to 38·4 c.c./min., and the average for all the adults taken together was 18·0 c.c./min. These figures are of the same order as those obtained by Ollayos & Winkler (1943). In the same person the clearances sometimes varied considerably from one time of day to another, and no satisfactory explanation can be advanced. In six out of the fifteen adult subjects it has been possible to compare the phosphate clearances during the forenoon with those during the afternoon, evening or night. In every instance the forenoon clearance has been the lowest, sometimes very much the lowest. Thus, the two forenoon clearances of no. 15 averaged 6·6 c.c./min. His afternoon clearance was 16·7 c.c./min. and his overnight clearance 20·1. No. 14's forenoon clearance was 6·5 c.c./min. and his evening clearance 19·5. The clearances of nos. 4 and 5 differed by similar amounts and those of the other two by smaller amounts. In this respect, therefore, the observations now being reported confirm those of Fiske (1921) and of Kleitman (1925).

The clearances of the newborn infants (Table 2) varied from 0·02 to 0·81 c.c./min., and if these figures are expressed per 1·73 sq.m. of surface area to make them comparable with those of the adults, the variations were from 0·18 to 6·64 c.c./1·73 sq.m./min. Thus, as in adults, the clearances showed great individual variations, but they were all lower than any of the adults and most of them were of quite a different order of magnitude. A reference to the way in which clearances are calculated shows that the higher serum levels in infancy as well as the lower urinary excretions combined to produce these differences. There is a suggestion from these results that the phosphate clearances may rise within a relatively short time of birth. Thus, no. 6's clearance rose from 0·77 c.c./1·73 sq.m./min. on the second day of life to 1·95 c.c. on the seventh, no. 7's from 1·67 c.c./1·73 sq.m./min. on the third day of life to 6·64 c.c. on the tenth day, and no. 9's from 0·18 c.c./1·73 sq.m./

min. on the fifth day to 2·93 c.c. on the fourteenth day. No. 10, who was 5 months old, had a clearance of 13·0 c.c./1·73 sq.m./min. which is close to the average figure for adults.

Table 1. The phosphate clearances of adults

No.	No. of periods averaged	Blood P (mg./100 c.c.)	Urine		
			P (mg./100 c.c.)	Volume (c.c./min.)	Clearance (c.c./min.)
1	1	2·6	37·0	1·18	16·8
2	5	4·7	106·0	3·03	21·0
3	6	3·7	30·3	3·78	17·6
4	2	3·1	22·1	1·77	11·7
5	2	3·1	23·0	1·44	8·9
6	2	2·3	30·5	2·16	28·1
7	1	3·6	87·0	0·70	17·0
8	2	3·4	66·5	1·14	20·5
9	1	4·1	39·0	1·14	10·9
10	1	4·6	112·0	1·33	33·0
11	1	3·8	33·0	4·46	38·4
12	1	3·2	80·0	0·95	23·6
13	4	4·8	48·3	1·66	16·2
14	3	4·4	65·0	1·1	14·7
15	4	4·2	37·4	1·21	12·5
					Average = 18·0

When data from more than one period have been available the figures given for the inorganic P in the urine, for the urine volume and clearance were all obtained by adding together the figures for the separate periods and dividing by the number of periods. The average figure shown at the bottom of the table was obtained in the same way.

Table 2. The phosphate clearances of infants

No.	Wt. at birth (g.)	Age (days)	No. of periods averaged	Blood P (mg./100 c.c.)	Urine vol. (c.c./1·73 sq.m./min.)	Clearance	
						c.c./min.	c.c./1·73 sq.m./min.
1	3250	4	6	6·8	3·71	0·68	5·25
2	3800	2	4	4·7	1·07	0·37	2·40
3	3200	11	4	6·4	2·56	0·21	1·77
4	2800	14	5	5·7	4·10*	0·53	4·28*
5	2950	4	1	3·9	0·24	0·29	2·41
6	3160	2	1	5·5	0·16	0·10	0·77
		7	1	6·9	0·98	0·26	1·95
7	2880	3	1	7·8	0·25	0·20	1·67
		10	1	6·7	0·36	0·81	6·64
8	2600	4	1	8·3	0·84	0·35	3·14
9	2650	5	1	6·2	0·75	0·02	0·18
		14	1	5·7	1·06	0·34	2·93
10		153	1	7·1	0·58	1·99	13·00

* The diuresis in this experiment was produced by giving 5 % glucose intravenously. Although no glucose appeared in the urine, the clearance of phosphate may have been reduced because the tubules were actively engaged in the reabsorption of glucose (Pitts & Alexander, 1944).

It is worth drawing attention to the very small quantities of P in the urine of some infants. 100 c.c. of no. 9's urine, for instance, contained only 1·22 mg. of inorganic P, and this is not a highly exceptional figure, for McCance & von Finck (1947) found that out of the seventy-two infants' urines, which were examined by them, three contained less than 0·1 mg./100 c.c. and twenty-five less than 1 mg./100 c.c. In comparison with these figures the present series of urines seem rich in phosphates and a more extended investigation might lower the average phosphate clearance for newborn infants very considerably. Walker & Hudson (1937) drew attention to the fact that the glomerular filtrates of Amphibia contained the same concentration of inorganic P as the plasma, but that the formed urine might occasionally contain a smaller one. This is evidently a common phenomenon in human infants, but it must be extremely rare or unknown in adults except perhaps after large doses of insulin when the excretion of P has been found to fall to levels as low as 0·7 mg./hr. (Blatherwick *et al.* 1924).

TABLE 3. The effect of urine flow upon the phosphate clearance

No.	Lowest minute volume (c.c./ 1·73 sq.m.)	Clearance (c.c./min./ 1·73 sq.m.)	Highest min. volume (c.c./ 1·73 sq.m.)	Clearance (c.c./min./ 1·73 sq.m.)
Infant 1, 1st exp.	0·45	2·30	2·46	7·05
Infant 1, 2nd exp.	4·60	5·40	6·60	6·00
Infant 2	0·61	2·30	1·85	3·70
Infant 3	0·11	0·51	8·60	4·80
Infant 4	0·80	2·00	9·72	6·35
Adult 2	0·50	16·50	10·00	24·00
Adult 3	1·00	14·20	8·90	19·00

Ollayos & Winkler (1943) found no correlation between the rate of formation of urine and the rate of excretion of P. The present results bearing upon this question have been assembled in Table 3, and they suggest that a correlation between these two variables certainly exists in infants, and may exist in adults. It is impossible to be more definite with the limited amount of data available.

DISCUSSION

The inulin clearances, i.e. the glomerular filtration rates, are now generally recognized to be much lower in infants than in adults (McCance, 1946). Hence the clearances of all substances such as urea which depend for their clearance upon the glomerular filtration rate must be expected to be lower in infants than in adults, and for urea this has been shown with certainty to be the case. Phosphates, sodium and chloride, however, are actively reabsorbed by the tubules. The glomerular filtrates always contain far more of these ions than the formed urine, and consequently the amounts excreted per unit of time depend largely upon the reabsorptive activity of the tubules. This may not be very great in infancy, for in some respects the tubules are known to be less effective at that age than they are in adult life (Dean & McCance, 1947). Owing to the low glomerular filtration rates, however, the tubules need not be so effective and are certainly effective enough in most instances, for it is truly remarkable how little P may remain in the urine after its passage through an infant's tubules. The same is true of sodium and chloride. Looked at it in one way, the low P clearances in infancy *may* be an indication of ill-matched tubular and glomerular function, i.e. of overactive tubular reabsorption, but it is quite evident that a great deal more work will have to be done before these problems can be regarded as solved.

SUMMARY

1. The phosphate clearances have been found to be much lower in newborn infants than in adults. The range in infants was from 0·18 to 6·64 c.c./1·73 sq.m./min., and in adults from 8·9 to 38·4 c.c./min.

2. The phosphate clearances in infancy probably vary with the rate of urine flow, and may rise to adult levels within a few weeks or months of birth.

3. In confirmation of previous work the phosphate clearances of adults were found to be at their lowest during the forenoon.

Most of the infants used for this investigation were born at the Landesfrauenklinik, Wuppertal, and the authors are very grateful to Prof. K. J. Anselmino and his staff for their help and co-operation. Four babies were obtained through other German hospitals and Prof. E. Thomas, Duisburg, Dr F. W. Koenen, Krefeld, and Dr J. Gehrt of Elberfeld have been particularly helpful. The oldest child was investigated at Cambridge through the co-operation of Dr Margaret Sutton.

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THE DIRECTIONAL AND SPECTRAL SENSITIVITIES OF THE RETINAL RODS TO ADAPTING FIELDS OF DIFFERENT WAVE-LENGTHS

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The marked dependence of visual response on the angle at which the rays forming the stimulus strike the retina is believed to be a property of cone vision. When conditions are such that the response to the stimulus is mediated by the retinal rods there is little or no variation with angle of incidence, except possibly for monochromatic stimuli in the orange and red. Experimentally, the angle of incidence is varied by converging the rays from the stimulus to a beam of very small cross-section in the plane of the fully adapted pupil and by directing this beam to different points in the pupil opening. If the same visual effect is produced by beam intensities I_c and I_e entering respectively at the centre and near the edge of the pupil, the ratio I_e/I_c is a convenient measure of the directional sensitivity of the retinal mechanism called into play. Measurements of the threshold intensity T for a small (1° square), flashing (0·063 sec. exposure), monochromatic test stimulus seen against a dark background at 5° in the parafovea by the completely dark-adapted eye show that the directional sensitivity T_e/T_c is nearly constant and equal to unity for wave-lengths λ below about 600 mμ. but increases to about 4 as λ is increased from 600 to 700 mμ. (Stiles, 1939). If, in this latter range, the rod mechanism of the retina were solely responsible for threshold vision of the test stimulus for both points of entry—as is certainly the case at shorter wave-lengths—it would be necessary to admit that the response of the parafoveal rods to red light was directional. More probably, however, the sensitivity of the parafoveal cones to a stimulus of the kind used becomes equal to, and then exceeds that of the parafoveal rods as the red end of the spectrum is approached, so that the directional property of the cones may be responsible for the observed directional effect in the red. The possibility of a directional response of the rods to red light would then remain open. In the present work this question is re-examined both by the original method and by a modified method.

The modified or indirect method uses the fact that for a test stimulus of a degree or more in diameter and of short wave-length, say 490 mμ., which is viewed parafoveally, the absolute threshold of the cone mechanism greatly exceeds—by a factor of 100 or more—the absolute threshold of the rod mechanism. This is borne out by the large difference between the achromatic and chromatic threshold, by the high value of the threshold observed a few minutes after intense light adaptation when only the cones have had time to recover (see particularly Wald, 1945), and by the position of the kink corresponding to the rod-cone transition in curves showing the variations of threshold with intensity level (see Stiles, 1939). It follows that if by any means the observed parafoveal threshold is raised above the value for the dark-adapted retina by a factor substantially below 100, then it must still be the rod mechanism which is responsible for vision of the test stimulus at the threshold. Suppose the threshold is raised by exposing the retina continuously to an extended and uniform adapting field of wave-length μ, centred on the parafoveal area at which the test stimulus of wave-length 490 mμ. is presented. The reciprocal of the intensity of the adapting field expressed in energy units, which is required for different wave-lengths μ to increase the threshold above the absolute value by some factor less than 100 defines the sensitivity of the rod-mechanism to the adapting effect of the field. The spectral sensitivity curve found in this way has been shown (Stiles, 1939) to be very similar in shape to that determined by the direct method. Further evidence on this point is given below. If now the *adapting field* is made to enter the eye as a narrow beam, the intensities necessary to raise the threshold by the fixed factor can be determined (*a*) when the beam enters at the centre, (*b*) when it enters near the edge of the dilated pupil, the point of entry of the test stimulus being kept the same throughout. The ratio of these intensities is a measure of the directional sensitivity of the rod mechanism to the adapting effect of light of wave-length μ. The advantage of the indirect method of determining the spectral and directional sensitivities of the rod mechanism is that it can be applied throughout the spectrum including the red end, with fair certainty that cone vision is not obscuring the results.

METHODS

The apparatus—similar in principle to that used by Stiles (1939) but rebuilt with certain improvements—needs only a brief description. Referring to Fig. 1 (*a*), a parallel beam from a double monochromator (F) passes through the lenses L_1 and L_2 which together form a system of zero power, and through the 2 in. beam-splitter cube C_1 to be brought to a focus in the plane of the apparent pupil of the subject's eye E, by the lens L_3. At the focus, the beam forms an image of the exit slit of the monochromator, of height 0·5 mm., width 0·19 mm., and this image can be moved to any position in the pupil, by a motion, in its own plane, of the larger lens of the zero power combination L_1L_2. The subject sees the lens L_3 as a uniformly illuminated, circular patch of 10° diameter—the adapting field. The test stimulus is obtained in just the same way from a second double monochromator T, the patch being limited to a 1° square by the diaphragm D_1 and turned through 90° by reflexion in the beam-splitter cube C_1. The image of the exit slit of T in the pupil plane has dimensions, height 0·5 mm., width 0·4 mm. approximately. A rotating shutter in the test stimulus beam exposes the latter for 0·2 sec. once in 3 sec., and also operates a warning buzzer which sounds 0·5 sec. before and 0·1 sec. after each exposure. The second 2 in. beam-splitter cube C_2

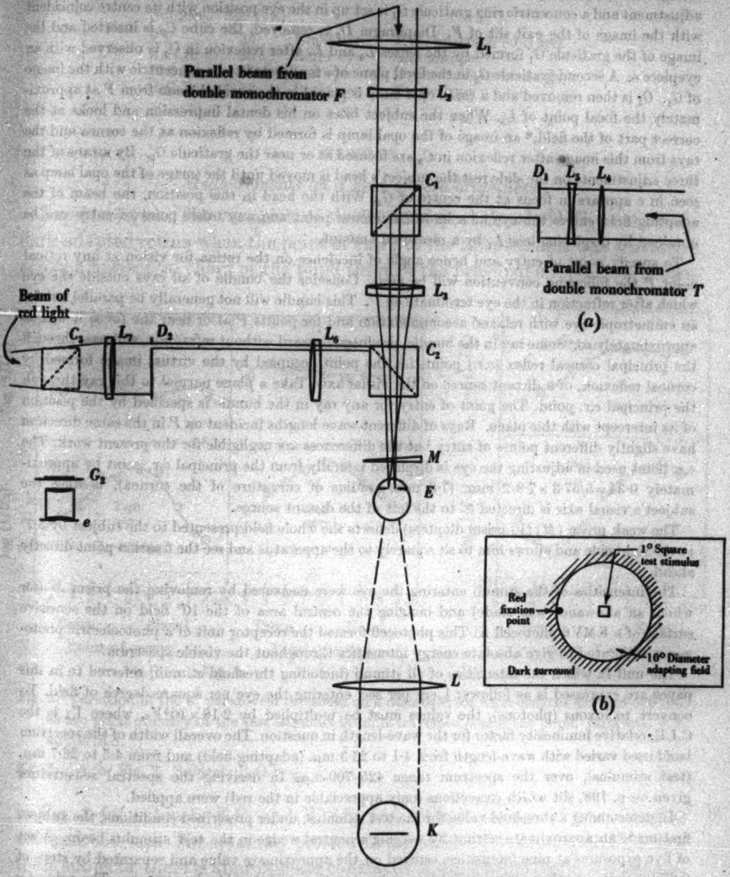
L_1
Parallel beam from double monochromator F
L_2
D_1
L_5
L_4
C_1
Parallel beam from double monochromator T
L_3
(a)
Beam of red light
C_3
L_7
D_2
L_6
C_2
M
G_3
e
E
1° Square test stimulus
Red fixation point
10° Diameter adapting field
Dark surround
(b)
L
K

Fig. 1. (*a*) Plan of apparatus (not to scale). (*b*) Observational field as seen by the subject.

introduces the fixation field of deep red, filtered light, again focused in the plane of the pupil. A diaphragm D_2 in the focal plane of lens L_6 limits the beam to the required fixation pattern which in the present work consists of a single small patch a few minutes in diameter placed 5° to the left of the test stimulus. Fig. 1 (*b*) shows the field as seen by the subject.

The subject's head is kept rigidly fixed with respect to the apparatus by requiring him to bite on a dental impression formed in sealing-wax on a brass base-plate carried by a massive lathe slide-rest equipped with adjustments in three directions and clamped to the table. The head is brought to the correct position in the following way. Lens L_1 is first moved to its standard 'zero' adjustment and a concentric ring graticule G_1 is set up in the eye position with its centre coincident with the image of the exit slit of F. Diaphragm D_2 is removed, the cube C_2 is inserted and the image of the graticule G_1 formed by the lenses L_6 and L_7 after reflexion in C_2 is observed with an eyepiece e. A second graticule G_2 in the focal plane of e is adjusted to be concentric with the image of G_1. G_1 is then removed and a small opal lamp is placed in the parallel beam from F at approximately the focal point of L_3. When the subject bites on his dental impression and looks at the correct part of the field,* an image of the opal lamp is formed by reflexion at the cornea and the rays from this image after reflexion in C_2 are focused at or near the graticule G_2. By means of the three adjustments on the slide rest the subject's head is moved until the image of the opal lamp as seen in e appears in focus at the centre of G_2. With the head in this position, the beam of the adapting field enters the eye at a known reference point and any other point of entry can be obtained by displacing lens L_1 by a measured amount.

To specify point of entry and hence angle of incidence on the retina for vision at any retinal point P the following convention will be used. Consider the bundle of all rays outside the eye which after refraction in the eye terminate on P. This bundle will not generally be parallel but for an emmetropic eye with relaxed accommodation and for points P at or near the fovea it will be approximately so. Some ray in the bundle, produced forward without refraction, will pass through the principal corneal reflex (c.r.) point, i.e. the point occupied by the virtual image formed by corneal reflexion, of a distant source on the visual axis. Take a plane normal to this ray through the principal c.r. point. The point of entry for any ray in the bundle is specified by the position of its intercept with this plane. Rays of different wave-lengths incident on P in the same direction have slightly different points of entry but the differences are negligible for the present work. The c.r. point used in adjusting the eye is displaced laterally from the principal c.r. point by approximately $0{\cdot}34 = 5/57{\cdot}3 \times 7{\cdot}8/2$ mm. (7·8 mm. = radius of curvature of the cornea), because the subject's visual axis is directed 5° to the left of the distant source.

The weak prism (M) (10 prism diopters) deflects the whole field presented to the subject by 5·7° to the right side and allows him to sit squarely to the apparatus and see the fixation point directly ahead.

The intensities of the stimuli entering the eye were measured by removing the prism M (for which an allowance was made) and imaging the central area of the 10° field on the sensitive surface of a KMV6 photocell K. This photocell formed the receptor unit of a photoelectric photometer calibrated to give absolute energy intensities throughout the visible spectrum.

The unit in which the intensities of all stimuli (including threshold stimuli) referred to in this paper are expressed is as follows: 1 erg per sec. entering the eye per square degree of field. To convert to luxons (photons), the values must be multiplied by $2{\cdot}18 \times 10^8 V_\lambda$, where V_λ is the C.I.E. relative luminosity factor for the wave-length in question. The overall width of the spectrum band used varied with wave-length from 4·1 to 23·3 mμ. (adapting field) and from 4·5 to 26·7 mμ. (test stimulus), over the spectrum range 425–700 mμ. In deriving the spectral sensitivities given on p. 198, slit width corrections (only appreciable in the red) were applied.

In determining a threshold value for the test stimulus, under prescribed conditions, the subject first made an approximate setting by varying a neutral wedge in the test stimulus beam. A set of five exposures at nine intensities, centred on the approximate value and separated by steps of 0·07 log unit, was then given, in random order, together with ten blank exposures. The subject's response 'seen' or 'not seen' to each exposure was recorded and a rough frequency of seeing curve constructed, from which the final threshold was read off as the intensity corresponding to 50%

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* As diaphragm D_2 is removed, the fixation point is not visible but accurate fixation is not necessary for the present purpose.

seeing chance. About 1 hr. before each set of measurements the pupil of the subject's observing eye was dilated with euphthalmine hydrochloride. The subject then remained in complete darkness until observations were commenced.

Four subjects were used:

Subject	Age (yr.)		
F.F.	32	Right eye	Hypermetrope + 1·5 sph.
K.H.S.	25	Right eye	Emmetrope —
W.S.S.	45	Left eye	Hypermetrope + 0·5 sph.
R.G.B.	19	Right eye	Emmetrope —

RESULTS

Measurements on a field of zero brightness

Pupil traverses. The variation of the 5° parafoveal threshold U_λ of the fully dark-adapted retina when the point of entry of the test stimulus was traversed across a horizontal chord of the pupil through the principal c.r. point, is shown in Fig. 2 for subjects F.F. and W.S.S. and for test stimuli of wave-lengths 490 and 650 mμ. The quantity plotted is log sensitivity, i.e. log $(1/U_\lambda)$. For these subjects, there is approximate constancy of the sensitivity for $\lambda = 490$ mμ. and for $\lambda = 650$ mμ., a well-marked directional effect with maximum sensitivity when the beam enters near the pupil centre. For subject R.G.B., on the other hand, it was found (*a*) that maximum sensitivity for a red test stimulus occurred when the stimulus entered near the nasal edge of the pupil, (*b*) that for the blue-green test stimulus, the sensitivity, while approximately constant

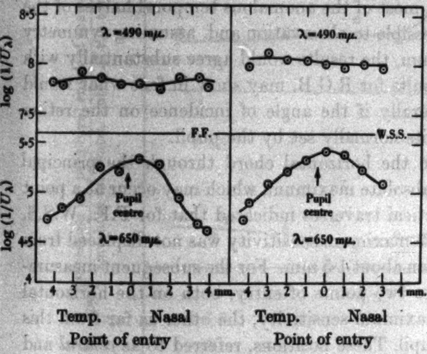

Fig. 2.

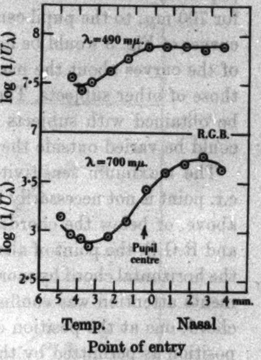

Fig. 3.

Fig. 2. Variation of the 5° parafoveal threshold on a dark field for wave-lengths 490 and 650 mμ. as the point of entry of the test stimulus beam is traversed across the pupil. Subjects: F.F. and W.S.S.

Fig. 3. As for Fig. 2. Subject: R.G.B.

in the nasal half of the pupil, dropped significantly in the temporal half (Fig. 3). Some difficulty was experienced in obtaining satisfactory measurements for this subject. The curves of Fig. 3 are two of several traverses all of which showed the features just noted. The rise in sensitivity near the temporal edge which appears in the curves of Fig. 3 is less certain. The fact that for some eyes the maximum sensitivity may occur for points of entry considerably displaced from the pupil centre has been noted before (Flamant, 1946; Stiles, 1939). Such displacements probably arise from a shearing-over of the retinal end-organs so that their axes are not pointing at the pupil centre. For R.G.B. this may well have occurred without any change in other threshold properties. His maximum sensitivity for $\lambda=490$ mμ. is about the same in absolute magnitude as the mean for F.F. and W.S.S. and other measurements show that at 650 and 700 mμ. it is only about 0·1–0·2 log units lower than the corresponding means for these subjects. Moreover, if one imagines R.G.B.'s pupil displaced about 3 mm. to the nasal side to bring the maximum sensitivity for 700 mμ. to the pupil centre, most of the anomalous temporal halves of the curves of Fig. 3 would be inaccessible to observation and, assuming symmetry of the curves about the maximum, the results would agree substantially with those of other subjects. The results for R.G.B. may show in fact what would be obtained with subjects generally if the angle of incidence on the retina could be varied outside the limits normally set by the pupil.

The maximum sensitivity on the horizontal chord through the principal c.r. point is not necessarily the absolute maximum, which may occur at a point above, or below the chord. Vertical traverses indicated that for F.F., W.S.S. and R.G.B. the point of absolute maximum sensitivity was not displaced from the horizontal chord by more than about 1·5 mm. For the subsequent measurements attention was confined to two points of entry, both on the horizontal chord, one at the position of maximum sensitivity, the other as far from this position as permitted by the pupil. These positions, referred to as *central* and *peripheral entry* respectively are shown in Table 1.

TABLE 1

	Points of entry used, mm. nasal (N.) or temporal (T.)	
Subject	Central entry	Peripheral entry
F.F.	0·6 T.	3·3 N.
	(later) 0·3 T.	4·1 T.
K.H.S.	0·3 T.	3·8 T.
W.S.S.	0·2 T.	3·2 T.
R.G.B.	2·4 N.	3·6 T.

The choice of positions for K.H.S., for whom no traverses were made, assumed that his maximum sensitivity occurred approximately at the pupil centre.

Two-point comparisons. Using the ratio of the threshold for peripheral and central entry as a measure of the directional sensitivity of the retina, the observations plotted in Fig. 4 show how the directional effect varies with the wave-length of the test stimulus. For the first three subjects the directional effect is slight and nearly constant for λ < 600 mμ., but between 600 and 670 mμ. it increases by 0·6–0·7 log units (4 or 5 to 1). For R.G.B. more results were obtained because of the wide spread in his observations. It seems probable that for this subject there is a significant rise in directional sensitivity in the range below 600 mμ. as well as a marked increase at longer wave-lengths.

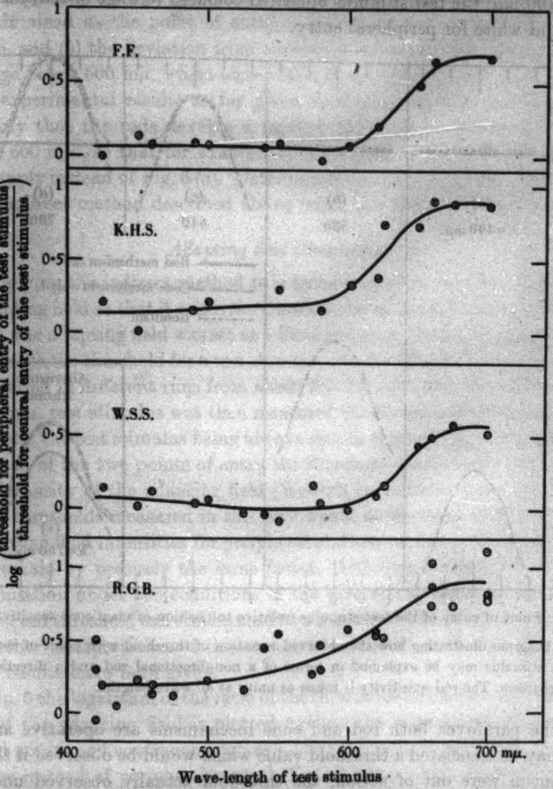

Fig. 4. Ratio of the 5° parafoveal thresholds for peripheral and central entry of the test stimulus in the pupil, for various wave-lengths of the test stimulus. Zero adapting field. Four subjects.

Subjects were asked to report on the apparent colour of the test stimulus when it was just above the threshold. The reports were fairly consistent in showing (*a*) that for wave-lengths below about 600 mμ. the test stimulus appeared white for both points of entry, (*b*) that as λ was increased from 600 to 700 mμ. the test stimulus appeared coloured (orange or red) for central entry and white for peripheral entry.

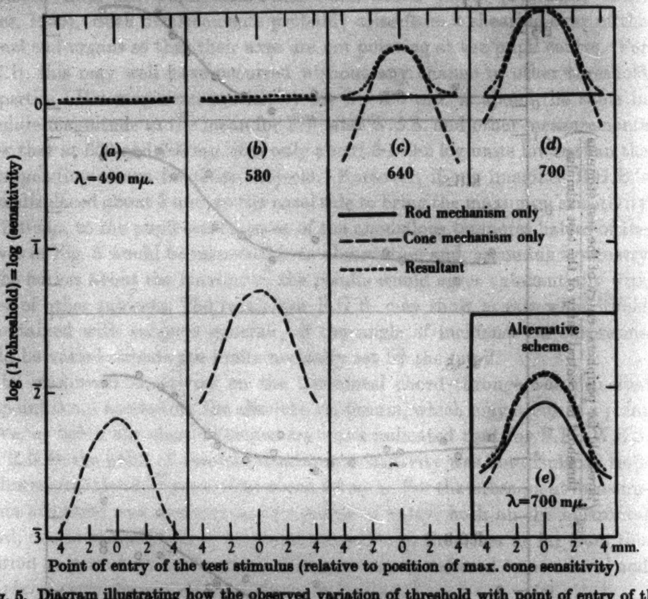

Fig. 5. Diagram illustrating how the observed variation of threshold with point of entry of the test stimulus may be explained in terms of a non-directional rod and a directional cone mechanism. The rod sensitivity is taken as unity at all wave-lengths.

In the parafovea both rod and cone mechanisms are operative and with each may be associated a threshold value which would be observed if the other mechanism were out of action, the threshold actually observed under any particular conditions being equal to the smaller of the two. The results just given are consistent with the view that, for all wave-lengths, the threshold of the rod mechanism is nearly independent of point of entry (at least within a range of 3–4 mm. from the central position) while for the cone mechanism the threshold has a pronounced minimum for central entry. The variation of observed threshold with point of entry for different wave-lengths would then arise in the way indicated in Fig. 5 (*a*) to (*d*) (log (sensitivity) = log (1/threshold) is plotted). Two points may be noted. Since, for peripheral entry, even a deep red stimulus is seen as white at the threshold it seems that cone sensitivity never sufficiently exceeds rod sensitivity completely to take over vision of the test stimulus at the threshold. The results for subject R.G.B. show that for him (and possibly for other subjects) (*a*) the constancy of the rod threshold is not maintained as the point of entry is moved to 6 mm. from the central position, and (*b*) the deviation from constancy increases with wave-length in the range below 600 mμ. where cone vision is not a factor.

The experimental results so far given would not exclude the alternative possibility that the rods develop a marked directional effect as λ increases beyond 600 mμ., so that for example at $\lambda = 700$ mμ. the scheme of Fig. 5 (*e*) would apply instead of Fig. 5 (*d*). Determinations of the directional sensitivity by the indirect method described above make this alternative improbable.

Adapting field illuminated

In applying the indirect method it is inconvenient to vary the intensity of the adapting field so that it raises the threshold by an exactly prescribed factor. Instead, the adapting field was set at a fixed intensity known from preliminary tests to raise the threshold for a test stimulus of wave-length 490 mμ. by a factor which varied in different runs from about 0·7–1·3 log units. The threshold for the 490 mμ. test stimulus was then measured for central and peripheral entry of the field, the test stimulus being always sent in through the central position. If for each of the two points of entry the threshold were strictly proportional to the intensity of the adapting field (Weber's law strictly true), the ratio of the two thresholds measured in this way would be identical with the ratio of the adapting field intensities for peripheral and central entry necessary to raise the threshold by precisely the same factor. Although Weber's law is a poor approximation under the conditions of the present experiments, the ratio of the observed threshold can still be used as a measure of the directional property. The further interpretation of this measure is deferred until after the experimental results have been given.

In Fig. 6 the logarithm of the ratio of the thresholds for central and peripheral entry of the adapting field is plotted against the wave-length of the latter (plotted points and continuous curves). The ratio central/peripheral is used in this case instead of peripheral/central (Fig. 4) since a high sensitivity to the adapting field corresponds to a high threshold value. Comparing the curves of Figs. 4 and 6, the outstanding differences is the absence of the marked increase in directional effect when μ exceeds 600 mμ. For the first three subjects, the directional sensitivity to the adapting effect of the field is small and nearly constant throughout the spectrum. For R.G.B. there is a rather larger directional effect, which increases with wave-length but the high values previously obtained in the red do not appear.

Although the adapting fields of different wave-lengths were set to raise the threshold for 490 mμ. by about the same factor, the field appeared much brighter as the red end of the spectrum was approached. Moreover in the red the apparent brightness increased materially when the point of entry was changed from the centre to the edge, despite the fact that the effect on the threshold for 490 mμ. was practically the same (except for subject R.G.B.).

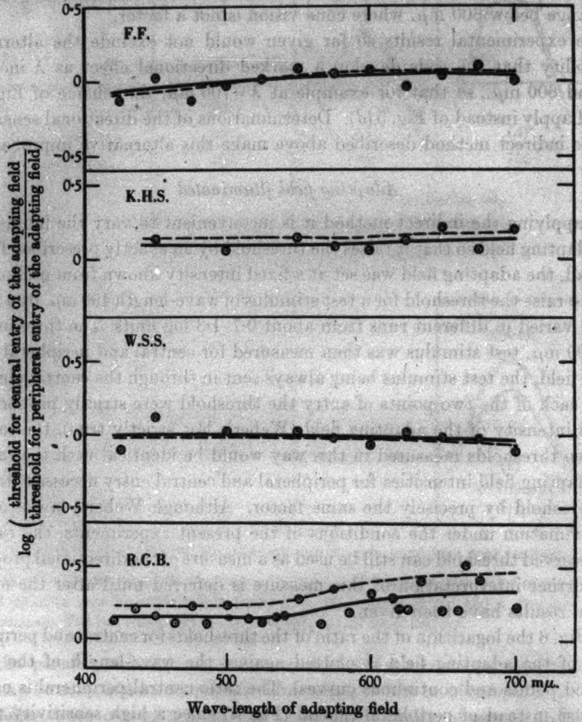

Fig. 6. Ratio of the 5° parafoveal thresholds for central and peripheral entry of the adapting field in the pupil, for various wave-lengths of the adapting field. Test stimulus of fixed wave-length (490 mμ.) and fixed point of entry (central) throughout.

The curve of Fig. 7 (taken from Stiles, 1939) shows how the threshold for a test stimulus seen by parafoveal rod vision increases as the intensity of the adapting field is raised. It was obtained under conditions which were the same as those used in the present work except that the test stimulus was exposed in flashes of 0·063 sec. instead of 0·2 sec. By convention, the units of test stimulus and field intensity in Fig. 7 are so chosen that the threshold for zero field, and the field intensity to raise the threshold by the factor 10, both have the value 1. The range of the present measurements corresponds to the shaded stretch of the curve of Fig. 7 where the mean gradient, *d* log (threshold)/*d* log (field), equals 0·7 as against the value 1 which would mean obedience to Weber's Law. The log ratios of the thresholds in Fig. 6 should therefore be increased by a factor of on the average 1/0·7 to obtain the correct measure of the directional sensitivity to the adapting effect of the field. This correction has been made in Fig. 6, where the broken curves are derived from the continuous curves by multiplying the ordinates by 1/0·7.

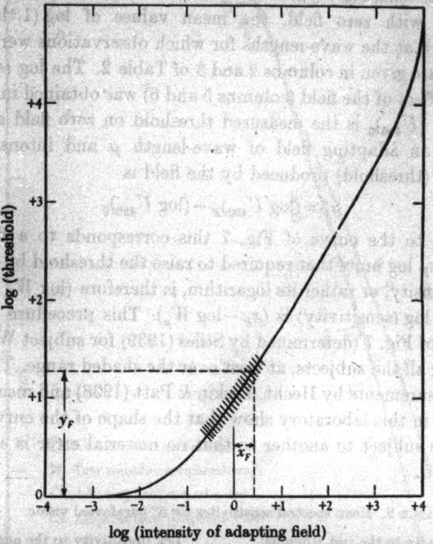

Fig. 7. Variation of the threshold of the parafoveal rod mechanism with the intensity of the adapting field (Stiles, 1939). The range involved in the present measurements is shown shaded.

It may be concluded that within 3–4 mm. of the central position the rod mechanism is nearly non-directional in its response to an adapting field for all wave-lengths of the latter. It is highly probable therefore that the rod response to a test stimulus is also non-directional throughout the spectrum, as it certainly is for λ below 600 mμ., and the scheme of Fig. 5 (*d*) must be accepted in preference to that of Fig. 5 (*e*).

For subject R.G.B., comparison of the curve of Fig. 4 with the broken curve of Fig. 6 in the range below 600 mμ. indicates that the deviations from non-directionality which the rod mechanism shows at 6 mm. from the central position are similar for test stimulus and adapting field.

Spectral sensitivity curves

As all the thresholds and field intensities were measured in energy units, certain spectral sensitivity curves can be derived from the results. For the measurements with zero field, the mean values of log (1/threshold), i.e. log (sensitivity), at the wave-lengths for which observations were made on all four subjects, are given in columns 2 and 3 of Table 2. The log (sensitivity) to the adapting effect of the field (columns 5 and 6) was obtained in the following way. Suppose $(U_{490})_0$ is the measured threshold on zero field and $(U_{490})_F$ is the value on an adapting field of wave-length μ and intensity $W\mu$. The increase in log (threshold) produced by the field is

$$y_F = (\log U_{490})_F - (\log U_{490})_0$$

and according to the curve of Fig. 7 this corresponds to a field intensity exceeding by x_F log units that required to raise the threshold by the factor 10. The latter intensity, or rather its logarithm, is therefore $(\log W_\mu - x_F)$ and the corresponding log (sensitivity) is $(x_F - \log W_\mu)$. This procedure assumes that the rod curve of Fig. 7 (determined by Stiles (1939) for subject W.S.S.) has the same *shape* for all the subjects, at least over the shaded range. The parafoveal threshold measurements by Hecht, Peskin & Patt (1938) and measurements on other subjects in this laboratory show that the shape of the curve varies very little from one subject to another so that no material error is expected from this assumption.

TABLE 2. Mean spectral sensitivites for 5° parafoveal vision

Log (sensitivity to the test stimulus) log $(1/U_\lambda)$			Log (sensitivity to the adapting field) $x_F - \log W_\mu$ (Derived from measurements with a test stimulus of wave-length 490 mμ.)		
Wave-length of test stimulus	Central entry	Peripheral entry	Wave-length of adapting field	Central entry	Peripheral entry
425	7·06	6·95	450	6·40	6·26
450	7·58	7·48	500	6·80	6·72
500	8·02	7·83	550	6·60	6·42
550	7·84	7·62	575	6·14	5·98
580	7·15	7·01	600	5·42	5·29
620	6·15	5·85	625	4·70	4·60
650	5·44	4·84	650	3·85	3·70
660	5·30	4·55	675	3·06	2·98
700	4·02	3·27	700	2·26	2·07
Additive constant to bring maximum sensitivity to unity	−8·06	−7·87		−6·84	−6·76

In plotting the four spectral sensitivity curves of Table 2, the maxima have been brought to the value unity by adding to the log values an appropriate constant which is also shown in Table 2. From the measurements of the

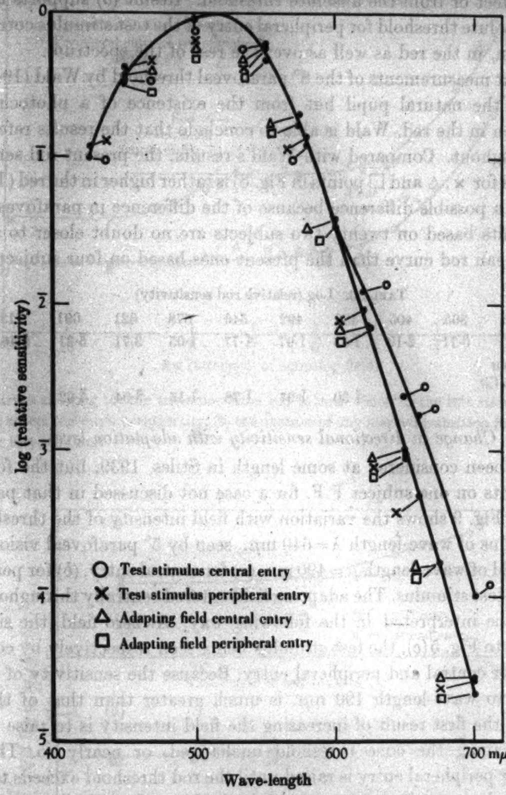

Fig. 8. The relative spectral sensitivity of the 5° parafoveal retina determined from the thresholds on zero field for central and peripheral entry of the test stimulus, and from the intensities of adapting fields, entering centrally and peripherally, which raise the threshold by a given factor.

directional effect already given it is clear that to a first approximation the test stimulus sensitivity curves (○ and × in Fig. 8) must agree for $\lambda < 600$ mμ. and then separate, while the adapting field sensitivity curves (△ and □) must agree over the whole spectrum. It is now seen (*a*) that for $\lambda < 600$ mμ. all four curves agree, (*b*) that for $\lambda > 600$ mμ. the adapting field curves agree with the test stimulus curve for peripheral entry. From (*a*), it follows that the relative spectral sensitivity of the rod mechanism is the same whether derived from the adapting effect or from the absolute threshold. Result (*b*) supports the view that the absolute threshold for peripheral entry of the test stimulus corresponds to rod vision, in the red as well as over the rest of the spectrum.

The recent measurements of the 8° parafoveal threshold by Wald (1945) were made with the natural pupil but from the existence of a photochromatic interval even in the red, Wald is able to conclude that the results refer to rod vision throughout. Compared with Wald's results, the present rod sensitivity curve (mean for ×, △ and □ points in Fig. 8) is rather higher in the red (Table 3). Apart from a possible difference because of the difference in parafoveal angle, Wald's results based on twenty-two subjects are no doubt closer to a representative mean rod curve than the present ones based on four subjects.

TABLE 3. Log (relative rod sensitivity)

Wave-length	365	405	436	492	546	578	621	691	713	750
Wald	$\bar{5}{\cdot}71$	$\bar{2}{\cdot}10$	$\bar{1}{\cdot}35$	$\bar{1}{\cdot}97$	$\bar{1}{\cdot}77$	$\bar{1}{\cdot}05$	$\bar{3}{\cdot}71$	$\bar{5}{\cdot}31$	$\bar{6}{\cdot}46$	$\bar{7}{\cdot}56$
Read from mean curve (×, △, □) Fig. 8	—	—	$\bar{1}{\cdot}30$	$\bar{1}{\cdot}94$	$\bar{1}{\cdot}78$	$\bar{1}{\cdot}15$	$\bar{3}{\cdot}94$	$\bar{5}{\cdot}62$	—	—

Change in directional sensitivity with adaptation level

This has been considered at some length in Stiles, 1939, but the following measurements on one subject F.F. for a case not discussed in that paper are of interest. Fig. 9 shows the variation with field intensity of the threshold for a test stimulus of wave-length $\lambda = 640$ mμ., seen by 5° parafoveal vision on an adapting field of wave-length $\mu = 490$ mμ. (*a*) for central entry, (*b*) for peripheral entry of the test stimulus. The adapting field entered centrally throughout. The curves can be interpreted in the following way. At zero field, the situation corresponds to Fig. 5 (*c*), the test stimulus being seen respectively by cone and rod vision for central and peripheral entry. Because the sensitivity of the rod mechanism to wave-length 490 mμ. is much greater than that of the cone mechanism, the first result of increasing the field intensity is to raise the rod threshold leaving the cone threshold unchanged, or nearly so. Thus the threshold for peripheral entry is raised until the rod threshold exceeds the cone threshold, it then remains constant until the field is sufficiently intense to begin raising the cone threshold. At this intensity the threshold for central entry should start to increase. On the whole the results fit this interpretation satisfactorily.

Precision of the threshold

The measured thresholds were all derived from rough frequency of seeing curves. The slope of the frequency of seeing curve at the 50 % value is a measure of the intrinsic sharpness or precision of the threshold, which must be regarded

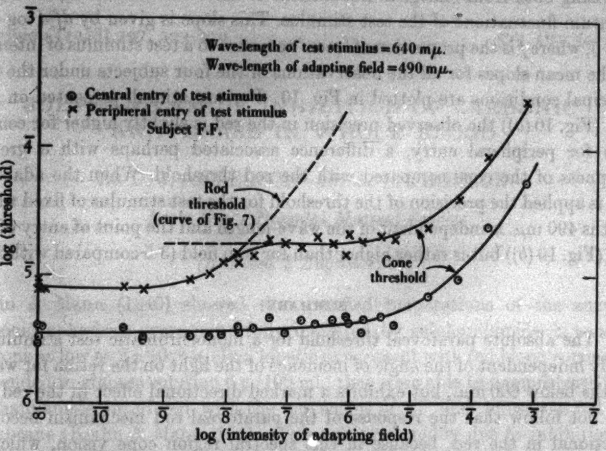

Fig. 9. Curves showing (*a*) the transition from rod to cone vision of the test stimulus when the latter enters the pupil peripherally, (*b*) the absence of any marked transition for central entry of the test stimulus.

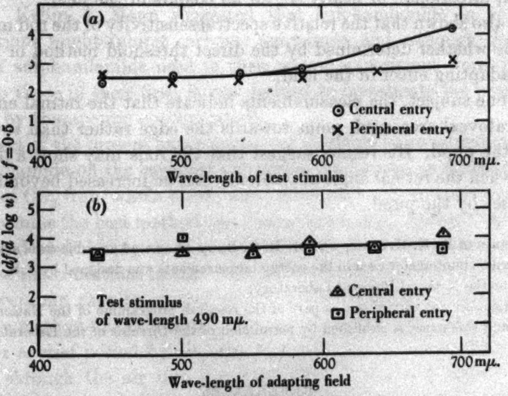

Fig. 10. Precision of the threshold. Slope ($df/d \log u$) of the frequency-of-seeing curve at the 50% point, for observations (*a*) on zero field with central or peripheral entry of the test stimulus, (*b*) on an adapting field entering centrally or peripherally.

as arising both from biological fluctuations of the visual mechanism and from quantum fluctuations of the test stimulus. This slope is given by $df/d \log u$ at $f=0{\cdot}5$, where f is the proportion of 'seen' response to a test stimulus of intensity u. The mean slopes for all the observations of the four subjects under the four principal conditions are plotted in Fig. 10. For test stimuli presented on zero field (Fig. 10 (*a*)) the observed precision in the red is slightly higher for central than for peripheral entry, a difference associated perhaps with a greater sharpness of the cone compared with the rod threshold. When the adapting field is applied the precision of the threshold for the test stimulus of fixed wave-lengths 490 mμ. is independent of the wave-length and the point of entry of the field (Fig. 10 (*b*)) but is rather higher than for zero field (3·5 compared with 2·5).

SUMMARY

1. The absolute parafoveal threshold for a monochromatic test stimulus is nearly independent of the angle of incidence of the light on the retina for wave-lengths below 600 mμ., but exhibits a marked directional effect in the red. It does not follow that the response of the parafoveal rod mechanism becomes directional in the red, because in this spectral region cone vision, which is highly directional, may be participating.

2. By determining the extent to which the threshold for a blue test stimulus ($\lambda=490$ mμ.) is raised by adapting fields of different wave-lengths, incident on the retina at different angles, it is shown that the rod response to an adapting field is non-directional, or nearly so, for all colours of the field.

3. It is also shown that the relative spectral sensitivity of the rod mechanism is the same whether determined by the direct threshold method or indirectly from the adapting effect of the field.

4. For one subject, the measurements indicate that the retinal end-organs, in the parafoveal area used, point towards the edge rather than towards the centre of the pupil. His results suggest that the rods may show a directional response when the retinal angle of incidence can be increased beyond the limit normally set by the pupil.

The assistance of Mr K. H. Spring and Mr R. G. Berry who acted as subjects is acknowledged. The photoelectric photometer used in the energy measurements was designed by J. S. Preston and constructed at the National Physical Laboratory.

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SULPHANILAMIDE INHIBITION OF CARBONIC ANHYDRASE IN WHOLE BLOOD

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Keilin & Mann (1940) showed that purified preparations of the enzyme carbonic anhydrase were powerfully inhibited by sulphanilamide in concentrations as low as 2×10^{-6} M. In a kinetic experiment with red blood corpuscles suspended in glucose solution 6 g./100 ml., they were able to demonstrate that sulphanilamide interfered with the movement of Cl^- ions across the red cell membrane (Keilin & Mann, 1941). This was presumably due to inhibition of the enzyme normally present within the red cells.

On repetition of the experiments in this laboratory it was found that the pH of the suspending fluid was approximately 9·5, and it was felt that an experiment designed to test carbonic anhydrase inhibition by sulphanilamide under conditions nearer those in the body would be of interest. The original results of Keilin & Mann suggested that sulphanilamide in therapeutic concentrations might inhibit carbonic anhydrase, and although the concentrations of sulphanilamide used in their second experiment were in striking contrast to those in their first, it was decided to investigate the possibility of inhibition by therapeutic concentrations.

The object of the present experiments was therefore to investigate the effects of sulphanilamide on the carbonic anhydrase mechanism as measured by the evolution of CO_2 from whole blood, and preliminary experiments were carried out to determine the best method and dosage to employ. At first CO_2 evolution was obtained by bubbling air through the blood, but it was found difficult to standardize the conditions in this type of experiment, because the bubble size and rate were not easy to control, and it was therefore abandoned. In broad outline, the method finally adopted was one in which freshly drawn blood was aerated for a brief period by allowing it to fall in small drops from a closed container through the air into liquid paraffin. The period allowed for CO_2 liberation could be varied by adjusting the length of the fall. Plasma CO_2 content, estimated with a Van Slyke volumetric apparatus before and after exposure, was used as a measure of CO_2 loss.

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Preliminary experiments were also carried out to determine the range of inhibitory concentrations of sulphanilamide in vitro. For this purpose a technique was used similar to that described by Meldrum & Roughton (1934). Blood was obtained by skin puncture and diluted 1:10 with various sulphanilamide solutions. 0·1 ml. of this blood-sulphanilamide solution was added to 1 ml. of 0·2 M.-$NaHCO_3$ solution in the reaction tube, and the evolution of CO_2 following the addition of 0·8 ml. of phosphate buffer pH 6·8 was measured. The volume change recorded on a manometer at 15 sec. intervals was taken as a measure of CO_2 evolution. A saturated solution of sulphanilamide containing 0·5 g./100 ml. was the first used, and this was equivalent to 5·0 g./100 ml. of whole blood. Sulphanilamide concentrations equivalent to 3·75, 2·5, 0·5 and 0·05 g./100 ml. of whole blood all inhibited carbonic anhydrase, but 0·005 g./100 ml. failed to produce inhibition. Results of this experiment coincided with those of Davenport (1945). It was therefore decided to begin testing inhibition in whole blood with not less than 50 mg. of sulphanilamide per 100 ml.

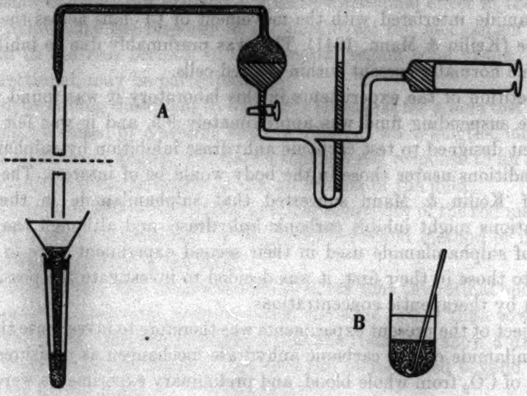

Fig. 1.

METHOD

Blood was taken from the cephalic vein of a volunteer into an apparatus similar to that described by Peters & Van Slyke (1932), which ensured collection without contact with air. The required amount of sulphanilamide was weighed into small containers (Fig. 1 B), and followed by 1 drop of 0·9 g./100 ml. NaCl so that a thick paste was formed. A layer of liquid paraffin was then added, and since the blood-collecting apparatus was graduated, a measured volume of blood could be delivered into the container beneath the paraffin (see Fig. 1 B). Solution of sulphanilamide was achieved by stirring for a few minutes with a glass rod without breaking the paraffin seal. The containers were then corked, leaving no air space. Because liquid paraffin is an inefficient seal against CO_2 loss, where possible every vessel containing blood or plasma under paraffin was tightly corked in this way.

Most subjects provided 100 ml. of blood, so that from each ten separate samples were obtained. Blood samples were transferred into a delivery apparatus, the details of which may be seen in Fig. 1 A. Constant speed of drop formation was achieved by displacing the blood with mercury injected from a syringe operated by a constant-speed motor. The effect of cross-draughts on the falling drops of blood was practically eliminated by allowing them to fall down a vertical tube 1 in. in diameter, at the bottom of which was placed a collecting funnel and centrifuge tube filled with liquid paraffin under the surface of which the blood dived on arrival. Two dropping tubes were used, one 8 ft. and the other 30 ft. high. Plasma was drawn off in modified Hempel pipettes. All experiments were conducted at room temperature and heparin (Vitrum), 5 units/ml. of blood, was the anticoagulant. One of the heparin preparations used was found to have an inhibitory effect on carbonic anhydrase. This was first demonstrated with the method described in this paper, and was confirmed with the use of the Roughton technique. The fact that pure preparations of heparin, however, caused no inhibition shows that the inhibitor in this preparation was an impurity.

Sulphanilamide concentration. The plasma sulphanilamide concentration was estimated by the method of Rose & Bevan (1944). The estimations were carried out with plasma for greater accuracy, because it was thought that, owing to poor solubility, samples of whole blood taken for analysis might contain undissolved sulphanilamide. These undissolved particles would, however, be thrown down by the centrifuge, so that the plasma estimation would give a figure for dissolved sulphanilamide only. Experiments were conducted to discover the distribution of sulphanilamide between cells and plasma, and it was found that with a plasma concentration of X mg./100 ml., the whole blood level was $X \times 12{\cdot}4/10{\cdot}2$ mg./100 ml. This relationship was established with concentrations of about 20 mg./100 ml., and it was not certain that it would be maintained at higher sulphanilamide levels. It was therefore decided not to convert observed plasma figures into whole blood figures since the plasma figure was known to be correct.

Time of exposure. The total time of exposure of the blood to air in the 8 and 30 ft. tubes was respectively 2·9 and 3·8 sec. This time included that taken in formation of the drop, its passage through the air and its spreading and submergence beneath the liquid paraffin. Experiments to separate the total time into its three components gave the results shown in Table 1.

TABLE 1. Separation of the total exposure time (sec.) into three components

	8 ft. tube	30 ft. tube
Time of formation	1·13	1·02
Passage through air	0·97	1·76
Spread and submergence	0·80	1·02
Total time	2·90	3·80

RESULTS

Addition of sulphanilamide without exposure to air. Table 2 shows the effect of a simple addition of an average of 60 mg./100ml. of sulphanilamide to venous blood. Plasma CO_2 content with and without sulphanilamide is shown in columns 1 and 2, and the differences are given in column 3. With '*t*' at 0·25 and 1 in 20 taken as the level of significance, these differences are probably due to random fluctuations. Thus the addition of sulphanilamide to venous blood without subsequent exposure to air does not alter its plasma CO_2 content.

pH *effect.* Even though the simple addition of sulphanilamide to blood had been shown to have no effect upon the plasma CO_2 content (Table 2), it was thought desirable to measure the blood pH change occurring on the addition of sulphanilamide. After the addition of 70 mg.% the pH of the whole blood changed from 7·45 to 7·41, and with 120 mg.% it changed to 7·47. These figures were obtained with a glass electrode and the change was not considered to be significant.

TABLE 2. The effect of the addition of sulphanilamide upon the CO_2 content of venous blood plasma

Vol. % CO_2 of venous samples

Sulphanilamide added	No sulphanilamide added	Δ	Plasma sulphanilamide concentration (mg. %)
57·5	64·2	−6·7	64.1
59·2	61·3	−2·1	61·9
65·2	59·5	+5·7	64·7
61·1	54·6	+6·5	64·7
67·6	69·0	−1·4	65·9
69·5	72·8	−3·3	55·5
69·7	69·8	−0·1	53·3
68·2	67·5	+0·7	58·3
65·4	67·9	−2·5	58·0

Average difference −0·36. σ difference ±1·406. $t=0·25$. Probability >0·80.

Effect of exposure after the addition of sulphanilamide. Table 3 shows the results of an exposure of 2·9 sec. without the addition of sulphanilamide to the blood. The average difference of +3·51 vol. between columns 1 and 2 indicates that the method allows liberation of CO_2 from venous blood in quantities approaching those liberated in the lungs. Again, without the addition of sulphanilamide, Table 4 shows the plasma CO_2 content of blood exposed for 2·9 and 3·8 sec. respectively. The average difference of +2·68 vol.% indicates that more CO_2 is lost when the exposure time is increased, thus demonstrating the kinetic nature of these experiments.

TABLE 3. Diminution of the CO_2 content of venous blood plasma after an exposure of 2·9 sec.

Vol. % CO_2 of venous sample

Not exposed	Exposed	Δ
72·2	67·0	+5·2
70·7	68·1	+2·6
68·7	68·3	+0·4
70·5	66·6	+3·9
69·4	68·3	+1·1
66·8	62·9	+3·9
65·2	60·7	+4·5
70·1	63·6	+6·5

Average difference +3·51. σ difference ±0·725. $t=4·84$. Probability <0·01.

TABLE 4. Difference between CO_2 content of venous blood plasma after exposure for 2·9 sec., and that after exposure for 3·8 sec.

Vol. % CO_2 of venous sample

2·9 sec.	3·8 sec.	Δ
66·5	63·9	+2·6
66·5	63·2	+3·3
65·8	63·4	+2·4
65·0	62·6	+2·4

Average difference +2·68. σ difference ±0·359. $t=7·45$. Probability <0·01.

Table 5 shows that, after exposure of both samples of blood to air for 2·9 sec., the plasma CO_2 content of blood, to which sulphanilamide has been added, is higher than that of blood containing no sulphanilamide. The average difference of +2·88 vol. % indicates that the speed of evolution of CO_2 from the blood in the presence of sulphanilamide is less than in untreated blood. This is probably due to the inhibitory action of the sulphanilamide on the carbonic anhydrase enzyme which catalyses the reaction,

$$H_2CO_3 \rightleftharpoons H_2O + CO_2.$$

TABLE 5. Difference between plasma CO_2 content of blood containing more than 50 mg. sulphanilamide per 100 ml. and plasma CO_2 content of blood containing no sulphanilamide. 2·9 sec. exposure

Vol. % CO_2 of venous sample			
Sulphanilamide added	No sulphanilamide added	Δ	Plasma sulphanilamide concentration (mg. %)
66·7	62·9	+3·8	73·8
64·3	60·7	+3·6	62·4
67·7	63·6	+4·1	52·0
68·1	66·6	+1·5	57·3
67·3	66·6	+0·7	50·2
68·1	64·5	+3·6	57·2

Average difference +2·88. σ difference ±0·580. $t=4·97$. Probability <0·01.

The average plasma sulphanilamide concentration in this series was 58·8 mg. % and with similar concentrations it was not found possible to demonstrate inhibition with the longer exposure time of 3·8 sec. However, with higher sulphanilamide concentrations inhibition was again shown. Table 6 demonstrates the differences in plasma CO_2 content of sulphanilamide and non-sulphanilamide containing blood, which were both exposed for 3·8 sec. Here the average difference is $+1·43 \pm 0·266$ giving a probability of $<0·02$, which clearly indicates inhibition. Table 7 shows that the plasma CO_2 content of blood containing an average of 16·7 mg. of sulphanilamide per 100 ml. of plasma, is not significantly different from that of blood containing no sulphanilamide, following exposure of both for 2·9 sec.

TABLE 6. Comparison between plasma CO_2 content of blood containing more than 100 mg. of sulphanilamide per 100 ml. and blood containing no sulphanilamide. Both exposed for 3·8 sec.

Vol. % CO_2 of venous sample			
Sulphanilamide added	No sulphanilamide added	Δ	Plasma sulphanilamide concentration (mg. %)
65·4	64·2	+1·2	105·1
64·7	63·7	+1·0	109·0
66·4	64·2	+2·2	104·3
65·8	64·5	+1·3	107·3

Average difference +1·43. σ difference ±0·266. $t=5·36$. Probability <0·02.

TABLE 7. Difference between plasma CO_2 content of venous blood plasma containing about 17 mg. sulphanilamide per 100 ml. and plasma CO_2 content of blood containing no sulphanilamide. 2·9 sec. exposure

Vol. % CO_2 of venous sample Sulphanilamide added	No sulphanilamide added	Δ	Plasma sulphanilamide concentration (mg. %)
69·4	72·4	−3·0	15·2
69·8	70·2	−0·4	16·0
70·7	71·3	−0·6	17·0
73·3	72·6	+0·7	17·1
71·7	72·9	−1·2	17·3
71·7	72·2	−0·5	17·3

Average difference −0·833. σ difference ±0·512. $t=1·63$. Probability >0·10.

DISCUSSION

These experiments indicate that sulphanilamide inhibition of carbonic anhydrase may be obtained in whole blood, if the concentration of the drug is sufficiently great, and the question must arise as to whether therapeutic concentrations of sulphanilamide acting in a normal lung could also inhibit the enzyme.

In answer, it may be said that therapeutic concentrations did not inhibit in these experiments. As shown in Table 7, no inhibition was obtained with plasma levels of sulphanilamide of an average of 16·7 mg./100 ml. This is of the same order of concentration as was used to derive the equation relating whole blood to plasma concentration, so that it was legitimate to calculate the whole blood concentration corresponding to this plasma level. According to the equation the plasma level of 16·7 mg./100 ml. was equivalent to a whole blood level of 21·5 mg./100 ml. and this, so far as has been ascertained, is above the maximum recorded therapeutic concentration. Hawking (1945) gives 4 mg./100 ml. as a usual concentration for therapeutic purposes, whilst Speert (1938) records a value of 7·6 mg./100 ml. after a large single dose. Marshall, Emerson & Cutting (1937) record blood levels of up to 11 mg./100 ml. with single doses and a maximum of 20·2 mg. following prolonged administration of the drug. Thus, in these experiments, therapeutic concentrations do not inhibit the enzyme.

On the other hand, although an attempt has been made to investigate the inhibition by sulphanilamide of the enzyme under physiological conditions, and although a liberation of about 3–4 vol. % of CO_2 has been achieved (a figure similar to that liberated from the blood in the lungs) the present experimental conditions differ in several important respects from those in the lungs. While on the one hand the CO_2 pressure gradient from blood to air is more favourable, and the exposure time is longer than in the lungs, the surface area per unit volume of a drop of blood cannot be as large as that offered by the lung capillaries. Furthermore, since the experiments were conducted at room temperature, the catalysed evolution of CO_2 must have been slowed down.

Inhibition has been shown in the presence of this temperature slowing with large concentrations of sulphanilamide and it might be that in the lungs therapeutic concentrations would inhibit the enzyme.

Against this it can be said that inhibition was only obtained when the sulphanilamide concentration was greater than 50 mg./100 ml. and we conclude that this was only just sufficient drug to show the effect, since a higher concentration had to be used in order to show inhibition with the longer exposure time of 3·8 sec. This being the case it seemed unlikely that the conditions existing in a normal lung, different from those of these experiments, could increase the efficiency of sulphanilamide so greatly that a concentration of as little as 10–15 mg./100 ml. could inhibit the enzyme, and one can say that the effect of therapeutic concentrations of the drug on CO_2 liberation in man is probably negligible.

SUMMARY

1. A method has been described in which whole blood can be aerated for short intervals of time.

2. By using this method, delay in the liberation of CO_2 from blood containing over 50 mg./100 ml. of sulphanilamide has been shown, and it is probable that this is due to inhibition of the enzyme carbonic anhydrase.

3. The question of inhibition by therapeutic concentrations of sulphanilamide is discussed.

Our thanks are due to many volunteers who have provided in all about 10 l. of fresh blood: N.G.B., D.G., L.A.W., P.T.C.R., P.R., G.A.D.H., A.S., G.P.A.S., P.M., N.S.G., E.O.H., D.S., J.M.H., J.V.K., C.J., J.G.P.H., D.J.S., D.G.A.W., P.R.H., P.W.R. Our thanks are also due to Mr J. D. Oriel who was responsible for some of the exploratory work and who provided a considerable quantity of blood.

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THE STIMULATING ACTION OF PHOSPHATE COMPOUNDS ON THE PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT

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In the experiments reported in this paper it has been shown that adenosine triphosphate (ATP) and creatine phosphate (CrP) as well as a number of simpler organic and inorganic phosphate compounds stimulate the perfused superior cervical ganglion in the cat. These experiments were undertaken because of the following two observations: (1) ATP, like some other phosphates, causes contractions of skeletal and smooth muscles (Buchthal, Deutsch & Knappeis, 1944, 1946; Buchthal & Folkow, 1944; Buchthal & Kahlson, 1944, 1946). (2) ATP and CrP may take part in the synthesis of acetylcholine in nervous and non-nervous tissue (Nachmansohn & Machado, 1943; Feldberg & Mann, 1945; Torda & Wolff, 1946*a*; Feldberg & Hebb, 1947; Comline, 1946).

(1) Buchthal & Kahlson (1944) concluded that 'the energy-rich phosphate bond in the form of ATP is not only the immediate source of energy recharge but also the agent for the release of contraction'. ATP, applied with a micropipette to isolated bundles of fibres of frog muscle, initiated tetanic contractions and caused changes in birefringence. Although the total amounts of ATP used were extremely small, the concentrations in which it was applied in order to produce the effects were high. For instance, the amounts of ATP applied to isolated muscle fibres were only $1\cdot3$–$1\cdot7 \times 10^{-5}$ μg., but they were applied in volumes of 4–7×10^{-3} μg.^{3}, so that the concentration of the solution of ATP was about 1 in 300. Similar effects could be produced by inosine triphosphate and by adenosine diphosphate (ADP) and by adenylic acid plus pyrophosphate or plus CrP, but not by adenylic acid or by CrP alone. Pyrophosphate by itself produced contracture. However, tetanic contractions were obtained with another inorganic phosphate, triphosphate, although without pronounced changes in birefringence. On the mammalian muscle (tibialis anterior of the cat) close arterial injections of 0·4–4 mg. of ATP also caused tetanic contractions, but

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† Beit Memorial Research Fellow.

so did the injections of ADP, adenylic acid, pyrophosphate and triphosphate but not of orthophosphate. These effects occurred after denervation and curarization of the muscle. The smooth muscle of the cat's stomach and bladder contracted to ATP in doses of from 0·1 mg. upwards but not to other phosphates injected arterially. These last muscles therefore 'reacted just as specifically towards ATP as does thrice precipitated myosin in the experiments of Needham, Kleinzeller, Miall, Dainty, Needham & Lawrence (1942)'. The isolated guinea-pig's intestine preparation on the other hand contracted to pyrophosphate also.

If these actions of ATP on intact muscle or muscle fibres were related specifically to the metabolism of muscle, there would be no reason to expect ATP to have a stimulating effect on nervous structures. It might, however, initiate excitation in the same way as acetylcholine and potassium do. In that case it might also be expected that a structure such as the superior cervical ganglion would react to ATP, since the ganglion cell with its axon has many pharmacological properties in common with the motor endplate and its muscle fibre.

(2) The fact that ATP, as well as CrP, may take part in the synthesis of acetylcholine suggested the possibility of explaining the failure of synaptic transmission, which occurs in a perfused sympathetic ganglion after prolonged preganglionic stimulation, as an impairment of synthesis of acetylcholine at the preganglionic endings due to exhaustion of their store of ATP or CrP. On this assumption the addition of these substances to the perfusion fluid should restore synaptic transmission. This assumption, however, proved to be wrong. Neither ATP nor CrP injections affected the degree of fatigue in the perfused superior cervical ganglion.

We found, however, that both substances had a stimulating effect on the ganglion. A study of the effect was therefore made and evidence was obtained to show that it is most likely due to the injection of phosphate as such. Certain resemblances between the responses of the ganglion to citrate, potassium and phosphate indicate that their mode of action is similar. Their stimulating effects are not abolished by curare. Thus their action is readily distinguished from that of acetylcholine, which like preganglionic stimulation is abolished by curare.

A preliminary account of our main results has been published in the *Proceedings* of the Physiological Society (Feldberg & Hebb, 1946).

METHODS

In cats, under chloralose anaesthesia, the superior cervical ganglion was perfused with Tyrode's solution as described by Feldberg & Gaddum (1934). Eserine sulphate was sometimes added to the perfusion fluid in a concentration of 1 in 200,000. On several occasions, tests were carried out on a ganglion which had been denervated in an aseptic operation under ether anaesthesia 8–10 days earlier. In place of the high pressure reservoir used by Feldberg & Gaddum for maintaining a constant perfusion pressure, a micro Dale-Schuster perfusion pump was used as described by Kahlson & MacIntosh (1939). At the beginning of perfusion the pressure was kept between 100 and 140 mm. Hg, but usually it had to be increased from time to time during the later part of the experiment to maintain a venous outflow of about 0·2 c.c. (6 drops)/min. The perfusion fluid was warmed by surrounding the cannula with a hot water jacket, a method found to be more convenient than the more elaborate heating system previously used by Feldberg & Gaddum. For the electrical stimulation of the cervical sympathetic nerve a condenser discharge stimulator was used, the cervical sympathetic being placed in Brown-Garry's fluid electrodes as modified by Collison (1933). The frequency of stimulation was about 10/sec.

The drugs used for stimulation of the ganglion were injected in a volume of 0·2–0·4 c.c. Acetylcholine was used as the chloride and the amounts injected refer to the salt. ATP was prepared as Ba-salt by a modified method of Lohmann (1931); before use it was converted into the Na-salt. As the dose of ATP in the text has been expressed usually as ATP pyro-P it has to be multiplied by 1·5 in order to obtain the value of the total phosphate injected. CrP was kindly prepared for us by Dr P. Eggleton and Dr R. N. Smith as the synthetic Ba-salt and converted before use into the Na-salt. 12 mg. of the Ba-salt contain about 1 mg. of total labile phosphate expressed as CrP. Yeast adenylic acid was obtained from British Drug Houses; a sample of muscle adenylic acid was kindly supplied to us by Dr Malcolm Dixon. The acids were neutralized before use. In the text, the amounts of adenylic acid and of inorganic phosphate compounds are expressed usually in terms of their total phosphate content. The total dose of each compound can, however, be calculated from the following: 1 mg. of total phosphate is contained in 12 mg. adenylic acid, in 11·5 mg. sodium orthophosphate (Na_2HPO_4, $10H_2O$), in 7·1 mg. sodium pyrophosphate ($Na_4P_2O_7$, $10H_2O$) and in 5·1 mg. sodium triphosphate ($Na_5P_3O_{10}$, H_2O).

In order to examine the effects of drugs on a paralysed ganglion a few c.c. either of curare (1 in 10,000 or 1 in 8000) or of eserine sulphate (1 in 4000 to 1 in 1000) were slowly injected into the perfusion cannula near the ganglion while the volume of fluid displaced backwards from the arterial cannula was simultaneously withdrawn from the inflow tubing. In this way there was no increase in the perfusion pressure during the injection and a continuous perfusion with either curare or eserine solution for several minutes was ensured.

RESULTS

Organic phosphates and related compounds

ATP stimulates the sympathetic ganglion. Usually the ganglion becomes more sensitive to ATP with continued perfusion and on repeated administration. In Figs. 1–3, records are seen of the contractions of the nictitating membrane obtained by stimulation of the ganglion by 0·4 and 0·1 mg. ATP pyro-P. The contractions produced by the injection of ATP were often found to last longer than contractions of the same strength produced by acetylcholine (see Figs. 1 and 6). In many experiments, but not in all, there was also a longer latency with ATP than with acetylcholine between the moment of injection and the beginning of the contraction. The threshold dose for the stimulating action of ATP was about 0·1 mg. ATP pyro-P.

The injection of ATP often produced slight changes in the venous outflow from the ganglion. In a few experiments the outflow temporarily increased after the injection, in several others the outflow appreciably decreased, whereas in some no change occurred. When there was marked vasoconstriction it outlasted the stimulating effect on the ganglion and was sometimes accompanied by a decreased response of the ganglion to preganglionic stimulation.

CrP resembles ATP in its action on the ganglion. In sensitive preparations the threshold dose in terms of labile P is again about 0·1 mg.; this figure also represents the total P present in the injection. The effects of 0·4 and 0·8 mg. CrP are seen in Figs. 1 and 2. The effects on the venous outflow were as irregular as in the case of ATP.

Adenylic acid stimulates the ganglion. When the effects are compared with those of ATP so that the total amount of P given either as ATP or adenylic acid are the same, adenylic acid is about as active as ATP. There is no difference between muscle and yeast adenylic acid. In the experiments of Figs. 1 and 2 0·4 mg. ATP pyro-P (0·6 mg. total P) had a somewhat stronger effect than 0·4 mg. P of either muscle or yeast adenylic acid.

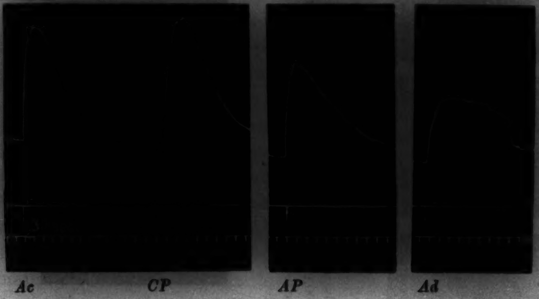

Fig. 1. Contractions of nictitating membrane during perfusion of denervated superior cervical ganglion with Tyrode's solution containing eserine 1 in 200,000. *Ac* = 0·2 μg. acetylcholine; *CP* = 0·4 mg. CrP-P; *AP* = 0·4 mg. ATP pyro-P; *Ad* = 0·4 mg. P of muscle adenylic acid. Time in 30 sec.

Adenosine and *creatine* were not found to have any stimulating effect on the ganglion (Fig. 2), although the doses given were equal to, or exceeded, the amounts of adenosine or creatine present in the doses of ATP or CrP respectively which produced strong effects on the ganglion.

Denervated ganglion. It is known that section of the cervical sympathetic nerve causes, within a few days, the disappearance of acetylcholine from the preganglionic endings and destroys their ability to synthesize it. Therefore any stimulating action on the ganglion which can be obtained a few days after its denervation cannot be attributed to a release of acetylcholine from the preganglionic endings and thus must be independent of the acetylcholine metabolism. In a ganglion so prepared the stimulating action of ATP, CrP and adenylic acid is retained, and the ganglion does not become less sensitive to these substances. For instance, the tracings of Figs. 1 and 2 were obtained

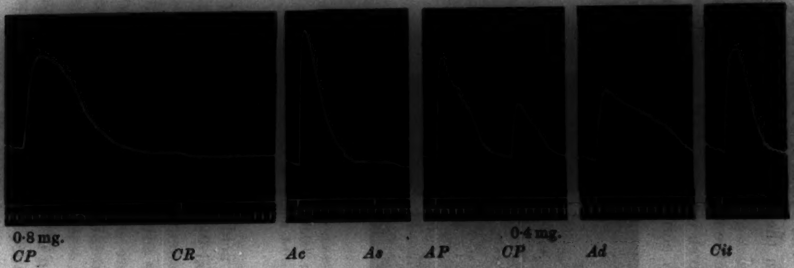

Fig. 2. Contractions of nictitating membrane during perfusion of denervated superior cervical ganglion with Tyrode's solution containing eserine 1 in 200,000. At *CP*=0·8 and 0·4 mg. CrP-P; *CR*=8 mg. creatine; *Ac*=0·4 μg. acetylcholine; *As*=8 mg. adenosine; *AP*=0·4 mg. ATP pyro-P; *Ad*=0·4 mg. P of yeast adenylic acid; *Cit*=0·45 mg. sodium citrate. Time in 30 sec.

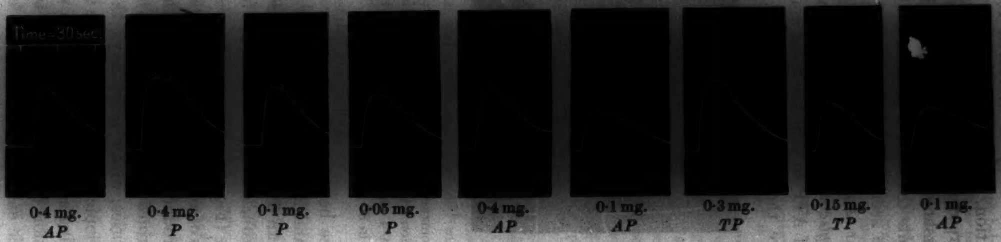

Fig. 3. Contractions of nictitating membrane during perfusion of superior cervical ganglion with Tyrode's solution containing eserine 1 in 200,000. *AP*=0·4 and 0·1 mg. ATP pyro-P; *P*=0·4, 0·1 and 0·05 mg. P of sodium pyrophosphate; *TP*=0·3 and 0·15 mg. P of sodium triphosphate. Time in 30 sec.

from ganglia perfused 8 days after section of the cervical sympathetic nerve when stimulation of the peripheral end had no effect on the nictitating membrane, showing that preganglionic denervation was complete. In view of these observations the stimulating action of the three organic phosphates must be independent of the acetylcholine metabolism in the ganglion.

Inorganic phosphates and citrate

Sodium pyrophosphate has a strong stimulating action on the ganglion (Fig. 3) and, in addition, causes a severe and prolonged vasoconstriction. When a large dose of pyrophosphate (over 5 mg.) was given, the outflow sometimes decreased from one drop in every 4 sec. to one drop in 5–10 min. It was possible that blocking the blood vessels with insoluble precipitated calcium salts of phosphate was in part responsible for this effect. The stimulating effect on the ganglion, however, preceded the decrease in venous outflow which was most marked after the relaxation of the nictitating membrane. After these large doses of pyrophosphate, the ganglion became insensitive to nerve stimulation or to other chemical stimuli during the period of decreased venous outflow and recovery was still incomplete even after an hour. When, however, smaller doses (1 mg. or less) were employed, they could be repeated at 5–10 min. intervals without depressing the ganglion. The effect of pyrophosphate in stimulating the ganglion is much stronger than that of ATP when the comparison is made on the basis of their phosphorus contents. For instance, in the experiment of Fig. 3 the effect of 0·4 mg. ATP pyro-P was approximately equivalent to 0·1 mg. P of pyrophosphate.

Sodium triphosphate acts like sodium pyrophosphate. On the basis of its total P it is about twice as active as ATP (Fig. 3).

Sodium orthophosphate has a stimulating and vasoconstrictor action. On the basis of its phosphate content orthophosphate is nearly twice as active as ATP in stimulating the ganglion. The effect of 4 mg. of sodium orthophosphate (i.e. about 0·35 P) corresponded to that of 0·4 mg. ATP pyro-P (i.e. about 0·6 mg. total P). Orthophosphate had a slightly stronger vasoconstrictor action than that of ATP.

Sodium citrate. Its stimulating action on the perfused sympathetic ganglion was demonstrated by Bronk (1939) who showed that citrate ions stimulate also the preganglionic nerve fibres in the ganglion. The action thus resembles that of potassium ions for which a similar action has been described by Brown & MacIntosh (1939). In our experiments, the threshold dose for the stimulating action of sodium citrate varied greatly (between 0·1 and 1 mg.) in different ganglia. The effect, like that of potassium or ATP, is not accounted for by the release of acetylcholine from the stimulated preganglionic nerve endings because it occurs also on the denervated ganglion (see Fig. 2). Small doses produced no changes in venous outflow but with large doses there was vasoconstriction.

Curare and eserine paralysis of the ganglion

A distinction may be made between substances which act on the perfused ganglion, like acetylcholine, the effect of which is confined to some part of the nerve cell, and those which act like potassium, the effect of which extends to the intraganglionic nerve fibres as well. Curare helps to differentiate between the two types of stimuli. It abolishes acetylcholine effects but not those produced by substances which act like potassium. On the other hand, a more complete and less specific paralysis of the ganglion and one which also abolishes the stimulating action of potassium can be produced by eserine in strong concentrations (Brown & Feldberg, 1936).

Curare. Its injection resulted in diminution in venous outflow due to increased vascular resistance and in profound depression or annulment of the effects of preganglionic nerve stimulation or of acetylcholine. When the perfusion fluid contained no eserine, curare abolished these effects, but when eserine was present curare usually did not abolish them so completely. In the case of nerve stimulation, the small contraction of the nictitating membrane which then still occurred was delayed; it began during the last few seconds of a period of nerve stimulation lasting 20 sec. or even after the stimulation had ceased. The curare, however, did not affect the sensitivity of the ganglion to ATP, CrP or citrate ions. For instance, in the experiment of Fig. 4, in which the ganglion was perfused with eserinized Tyrode's solution, the strong response to nerve stimulation and to 2 μg. of acetylcholine was nearly abolished by curare, but there was no change in the response to 0·3 mg. ATP pyro-P, and, when the dose of ATP was doubled, a stronger effect was obtained. In the experiment of Fig. 5, the ganglion was perfused without eserine. Curare completely paralysed the ganglion to nerve stimulation and to acetylcholine but ATP and citrate ions produced strong contractions which were comparable to those obtained before the addition of curare to the perfusion fluid and after it had been washed out. Similar results were obtained for the stimulating action of CrP. In one experiment, for instance, the injection of 0·4 mg. CrP-P gave only a small response which remained unaffected during perfusion with curare whilst a much stronger response to nerve stimulation was abolished.

Eserine. Very high concentrations reversibly paralyse the ganglion not only to acetylcholine or nerve stimulation but also to ATP, CrP or citrate.

In all experiments the perfusion of the ganglion was started with eserine 1 in 200,000; replacing a few c.c. of this fluid with a solution of eserine 1 in 10,000 was found to render the ganglion insensitive to acetylcholine or nerve stimulation but not to ATP, CrP or citrate ions, although the responses to these three substances were reduced. To annul their effects eserine had to be used in a concentration of 1 in 1000. The experiments were carried out on normal ganglia as well as on ganglia denervated 8–10 days before. For instance, the record shown in Fig. 6 was obtained from a denervated ganglion. At the

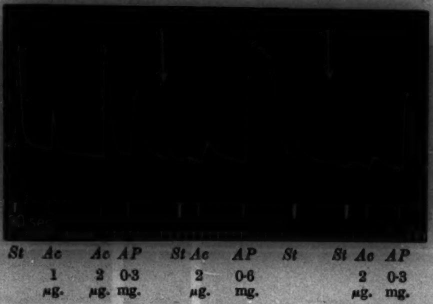

Fig. 4. Contractions of nictitating membrane during perfusion of superior cervical ganglion with Tyrode's solution containing eserine 1 in 200,000. At each arrow (↓) perfusion fluid replaced by 5 c.c. curare 1 in 10,000. *St* = 10 sec. stimulation of cerv.symp.; *Ac* = 1 and 2 μg. acetylcholine; *AP* = 0·3 and 0·6 mg. ATP pyro-P. Time in 30 sec.

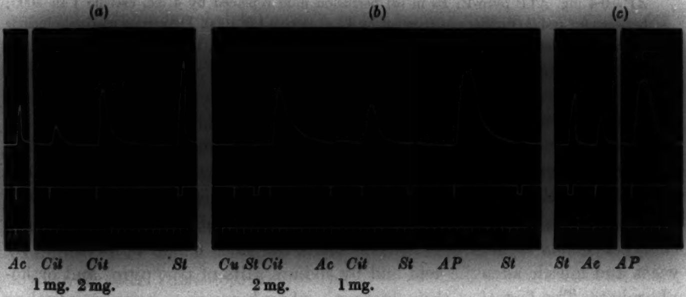

Fig. 5. Contractions of nictitating membranes during perfusion of superior cervical ganglion with Tyrode's solution before (*a*), during (*b*) and after (*c*) curare. *Cu* = perfusion fluid replaced by 7 c.c. curare, 1 in 8000, and washed out again between (*b*) and (*c*). *Ac* = 3 μg. acetylcholine; *Cit* = 1 and 2 mg. sodium citrate; *St* = 20 sec. stimulation of cerv.symp.; *AP* = 0·45 mg. ATP pyro-P. Time in 30 sec.

beginning of the experiment, in (*a*), strong contractions are produced by 1 μg. acetylcholine and by 0·4 mg. ATP pyro-P; at *Es* 4 c.c. of the perfusion fluid were replaced by an equal volume of a solution of eserine 1 in 2000; afterwards the effect of acetylcholine was abolished and that of ATP greatly reduced. At (*b*) another 4 c.c. of the perfusion fluid were replaced by an equal volume of a solution of eserine 1 in 1000; this stronger solution of eserine now also abolished the stimulating effect of ATP. Between (*b*) and (*c*) the strong solution of eserine was washed out and the response to acetylcholine and to ATP is seen to have returned.

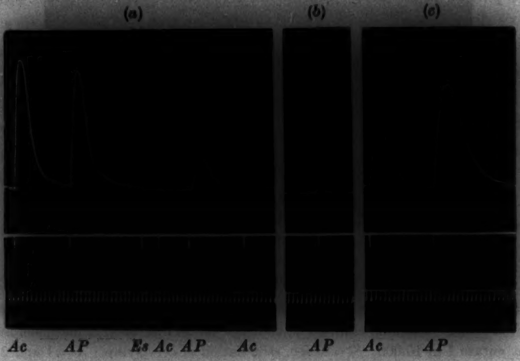

Fig. 6. Contractions of nictitating membrane during perfusion of denervated superior cervica ganglion with Tyrode's solution containing eserine 1 in 200,000. *Ac*=1 μg. acetylcholine; *AP*=0·4 mg. ATP pyro-P; *Es* in (*a*) perfusion fluid replaced by 4 c.c. eserine 1 in 2000; between (*a*) and (*b*) further replacement by 4 c.c. eserine 1 in 1000. The strong eserine solutions were washed out between (*b*) and (*c*). Time in 30 sec.

DISCUSSION

Our results show that ATP and CrP have a stimulating action on the perfused superior cervical ganglion of the cat. This action is exerted independently of the formation or release of acetylcholine from the preganglionic endings, since it is demonstrable on the denervated ganglion after degeneration of the preganglionic fibres. Further, the effect is independent of the presence of high-energy P bonds in the CrP and ATP molecules since it can be produced by yeast and muscle adenylic acid and by sodium salts of tri-, pyro- and orthophosphate. The fact that these inorganic phosphates may have an equal or even stronger stimulating action than the organic phosphates, together with the fact that neither creatine nor adenosine have any such action, leads us to conclude that in each case the effects are due to the injection of phosphate as such.

It is at present not possible to state definitely how the stimulating action of the various phosphate compounds on the perfused ganglion may be produced. The effect resembles that of citrate and potassium ions but not that of acetylcholine. This is shown by the fact that, unlike acetylcholine, neither the phosphate compounds, citrate nor potassium lose their stimulating effect on a ganglion paralysed by curare. The resistance to curare may be correlated with the ability of these substances to excite not only the ganglion cells but also the postganglionic fibres within the ganglion. On the other hand, paralysis of the ganglion by high concentrations of eserine affects also the response to citrate, potassium, ATP and CrP, probably because in this condition, the nerve fibre also has become inexcitable.

The calcium-binding properties of phosphate are well known, and are generally considered to be of importance in the aetiology of certain forms of tetany. By analogy, we may suspect that the action of the phosphate compounds on the superior cervical ganglion is due to these properties, and that stimulation occurs because the excess of phosphates rapidly lowers the concentration of calcium in the perfusate and extracellular fluid. Torda & Wolff (1946*b*) discussed such a mode of action for the muscle-contracting effects of ATP; Buchthal, Deutsch & Knappeis (1946), however, reject this explanation, because they found that the threshold concentration of citrate necessary for releasing contractions in skeletal muscles is much higher than that of ATP.

The interpretation of our results in relation to those of Buchthal and his co-workers on smooth and skeletal muscles presents a rather difficult problem. If the contractions produced by ATP on smooth muscles of the cat alone had to be considered, one might well come to the conclusion that they were correlated with a specific action of ATP. On the other hand, in its ability to contract mammalian skeletal muscle, or the smooth muscle of the guinea-pig's intestine, the action of ATP resembles more that found in the ganglion because ATP, on these tissues, can be replaced by more stable phosphate compounds. There are, however, some specific effects which ATP exerts even when injected arterially into a mammalian skeletal muscle, for instance, the pronounced changes in birefringence. This effect may depend upon the participation of ATP in some enzyme processes or topo-chemical reactions and is certainly related to its corresponding action on acto-myosin threads. In fact, Buchthal, Deutsch, Knappeis & Munch-Petersen (1947) have recently shown that of all phosphates examined, ATP alone was able to produce changes in birefringence on acto-myosin threads. There does not yet appear to be sufficient evidence to attribute the muscular contractions, produced by ATP injected arterially into a muscle, to a similar specific effect dependent on the high-energy P bond of the molecule. ATP has this action in common with many other substances, phosphates other than ATP, citrate and potassium. All these substances can initiate muscular contractions by direct effect on the muscle fibre, and their action is apparently not confined, as is that of acetylcholine and nicotine, to the motor endplate. In this there is a clear analogy with the stimulating effect of these substances on the sympathetic ganglion.

The inactivation of nervous tissue by extremely high concentrations of eserine has been made the foundation of the hypothesis that acetylcholine is intrinsically connected with the action potential of nerve membranes. In favour of this hypothesis, it has been stated that eserine, in extremely high concentrations, reversibly abolishes nerve action potentials (Bullock, Nachmansohn & Rothenberg, 1946); although Gilman (1946) as well as Bodansky, Tobias & Gerard (1947) have found that there is no quantitative relation between this effect of anticholin-esterases and their power of inhibiting the cholinesterase of nervous tissue. In our experiments on the perfused ganglion we have found that eserine in very high concentrations can be made to prevent the passage of a nerve impulse elicited by a variety of other agents, e.g. citrate, phosphate, acetylcholine, etc. This action can be demonstrated on the ganglion after degeneration of the preganglionic fibres as well as on the intact ganglion, although after denervation the ganglion no longer contains true cholinesterase (Sawyer & Hollinshead, 1945) or acetylcholine. Thus our results support those of Gilman and of Bodansky *et al.*, making it unlikely that the inactivation of nerve tissue by high concentrations of eserine is specifically related to its acetylcholine metabolism.

SUMMARY

1. Adenosine triphosphate (ATP) and creatine phosphate (CrP) have a powerful stimulating action on the perfused superior cervical ganglion of the cat. The effect is demonstrable also on the denervated ganglion after degeneration of its preganglionic fibres and therefore is independent of the acetylcholine metabolism of the preganglionic nerve endings.

2. ATP and CrP owe their stimulating action on the ganglion to the phosphate present in the molecule. The fact, however, that it is present as labile phosphate is apparently of no significance for this action. Equally strong effects could be obtained with yeast and muscle adenylic acid and with the sodium salts of tri-, pyro- and ortho-phosphate. Creatine and adenosine, on the other hand, had no stimulating effect.

3. The stimulating action of ATP and CrP on the perfused ganglion is abolished by eserine in very high concentration but not by curare. The effect therefore differs from that of acetylcholine but is analogous to that of potassium and citrate.

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THE SECRETION OF ADRENALINE FROM THE PERFUSED SUPRARENAL GLAND

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The experiments to be described were undertaken to study the formation and the output of adrenaline in the suprarenal gland when isolated and perfused. The perfusion of the suprarenal gland of the dog was described by Anitschkow & Kusnetzow (1928) and of the cat by Feldberg (1940); it has been more recently performed by Vogt (unpublished), whose technique we have followed very closely. We investigated the effect of changes in the oxygen supply produced by nitrogen and by injections of potassium cyanide on the output of adrenaline. We added adrenaline to the blood reaching the gland to see if adrenaline was stored. We found (*a*) that anoxia produced a discharge of adrenaline from the perfused gland, (*b*) that the amount of adrenaline secreted during splanchnic stimulation depended on the concentration of adrenaline in the arterial blood reaching the gland.

METHOD

A dog was anaesthetized with ether and 200 ml. blood was withdrawn from the carotid artery into heparin. After making up to 250 ml. with Ringer's solution, this blood was circulated by a Dale-Schuster pump through a Hooker oxygenator until the perfusion was begun. The blood was aerated with oxygen containing 5% CO_2. The ether anaesthesia of the dog was then replaced by nembutal given intravenously together with 200 ml. Ringer's solution. After evisceration and removal of the right kidney, the right suprarenal was excised, weighed, ground with sand and extracted with 10 ml. N/10-HCl. The extract was boiled and centrifuged. The adrenaline content was determined as described below. The vessels running dorsad behind the aorta and vena cava were tied from the bifurcation up to a point about 1 in. above the left suprarenal. The left kidney was removed. All vessels round the lateral periphery of the left suprarenal were divided until a length of both vena cava and aorta, with the suprarenal gland attached to it, was freed from its bed. An approach was made to the left splanchnic nerve through an intermuscular space in the diaphragm, and the nerve, after being divided in the thorax, was freed until close to the gland. Loose ligatures were placed round the vena cava and aorta above the gland, and artery clips were placed below. Cannulae were then inserted as shown in Fig. 1. The loose ligatures were tied and simultaneously the artificial circulation was begun. The dog was then bled out into heparin from the carotid artery, and this blood, diluted with Ringer's solution to 250 ml., was added to the pump, making a total initial volume of 500 ml. The blood was pumped from the oxygenator to a 4-way cannula inserted in the aorta. One side-arm was connected to a manometer, and the other led the bulk of the blood through a Starling heart-lung resistance back to the oxygenator. Thus the blood was in continuous circulation to minimize the formation of vasotonins. Dextrose (25 mg.) was added every 20 min. to maintain the blood sugar concentration at about 0·12%. The blood collected from the vein was not returned to the circuit, but was collected in successive portions which were at once centrifuged so that the adrenaline content of the plasma could be determined.

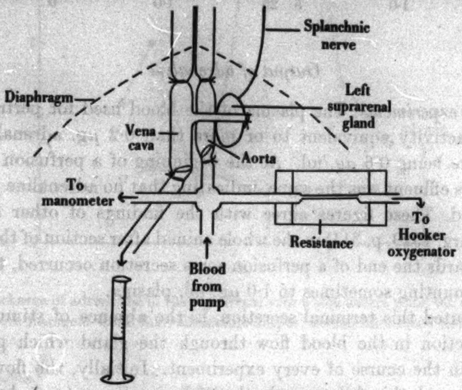

Fig. 1. Diagram of perfusion system.

Estimation of adrenaline. The estimation of adrenaline was performed on the isolated intestine of the rabbit, of which the duodenum and proximal portions were used. A known solution of adrenaline was freshly prepared as a standard. Each sample of plasma was estimated by two workers independently as soon after centrifugation as possible. When the splanchnic nerve was stimulated it was laid upon a shielded silver unipolar electrode 3 mm. wide. Condenser discharges from a neon lamp circuit were applied at the rate of 20 per sec. Accumulation of fluid in the neighbourhood of the electrode was prevented by a suction pump.

RESULTS

Contents of the two glands. The amount of adrenaline present in the two suprarenal glands of an animal has been shown by various observers to be approximately the same per g. of gland weight. Elliott (1912) found this to be true for cats. We have made some observations in dogs in which we compared the adrenaline present in the right gland removed at an early stage of the preparation, with the adrenaline present in the left gland after various periods of perfusion. This enabled us to discover if the change from the natural to the artificial circulation affected the adrenaline content. The results are shown in Table 1. They indicate that there was no difference in the adrenaline content even when one gland had been perfused for periods up to 5½ hr.

TABLE 1. Adrenaline content (mg./g.) of suprarenals

Right control	Perfusion period hr.	Perfusion period min.	Left (perfused)	Difference %
1·10	–	45	1·03	6
2·50	2	—	2·50	0
1·17	2	40	1·05	10
2·18	3	15	2·24	3
1·0	5	25	1·0	0

Output of adrenaline

In control experiments. The plasma of the blood used for perfusion always showed an activity equivalent to or more than 0·2 μg. adrenaline/ml., the highest figure being 0·6 μg./ml. At the beginning of a perfusion the amount in the venous effluent was the same, indicating that no adrenaline was secreted by the gland. These figures agree with the findings of other authors (see Trendelenburg, 1929, p. 314) in the whole animal after section of the splanchnic nerves. Towards the end of a perfusion some secretion occurred, the excess in the vein amounting sometimes to 1·0 μg./ml. plasma.

We attributed this terminal secretion, in the absence of stimulation, to a steady reduction in the blood flow through the gland which progressively diminished in the course of every experiment. Initially, the flow was about 1·5 ml./min. and this fell towards the end sometimes to as low a flow as 0·4–0·5 ml./min. Occasionally the perfusion started with a flow of 5·0–6·0 ml./min., but this high rate rapidly fell, and remained at about 1 ml./min.

Since the rise in the adrenaline content of the venous blood appeared to correspond to the decline in the flow it seemed likely that the adrenaline secretion was due to anoxia of the medulla.

Effect of anoxia. We therefore tested the effect of reducing the arterial oxygen saturation by aerating the blood with nitrogen containing 5% CO_2. (Normally oxygen with 5% CO_2 was used, as then the flow through the gland was found to be better than with oxygen alone.) Fig. 2 provides an example of what was found. After the perfusion had proceeded for 1 hr., the nitrogen-CO_2 mixture was substituted. The oxygen saturation of the blood was slowly reduced and 30 min. later the adrenaline secretion rose in stages up to 4 μg./ml. plasma. When the nitrogen was discontinued, the secretion returned to the previous value after a similar latent period.

The same influence of anoxia was demonstrated by experiments in which potassium cyanide was injected. Fig. 3 shows the sudden rise in adrenaline output after administration of 0·5 mg. KCN, and again 40 min. later after 2·0 mg. KCN. With each injection there was an increase in blood flow, followed, after the second injection, by a considerable reduction, and the adrenaline secretion was therefore calculated per min.

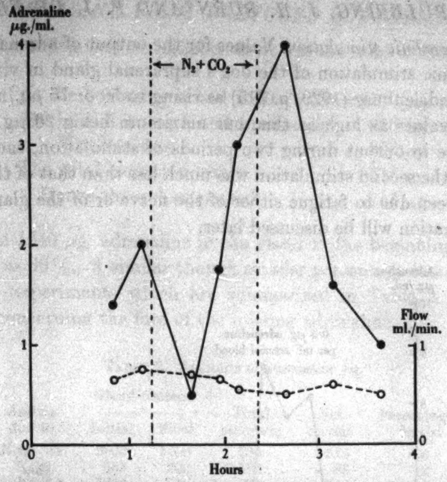

Fig. 2. The discharge of adrenaline in the suprarenal vein which occurs when the blood is aerated with nitrogen. Solid line: adrenaline output; dotted line: blood flow.

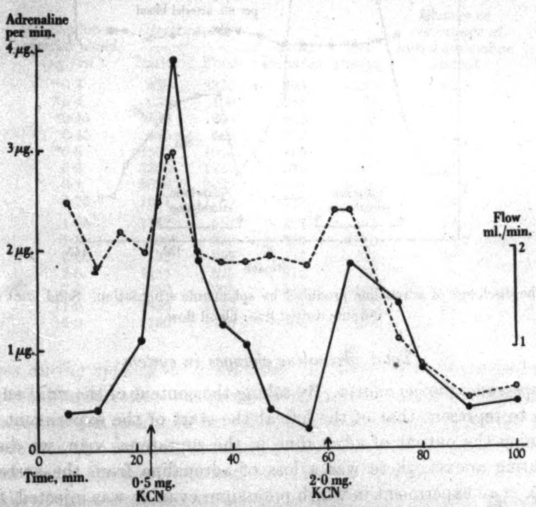

Fig. 3. The discharge of adrenaline produced by injection of KCN. Solid line: adrenaline output, dotted line: blood flow.

During splanchnic stimulation. Values for the output of adrenaline resulting from splanchnic stimulation of the dog's suprarenal gland in vivo have been given by Trendelenburg (1929, p. 315) as rising to 50 or 75 μg./min. We have not reached values as high as this, our maximum being 26 μg./min. Fig. 4 shows the rise in output during two periods of stimulation, each of 35 min. The effect of the second stimulation was much less than that of the first which might have been due to fatigue either of the nerve or of the gland. An alternative explanation will be discussed later.

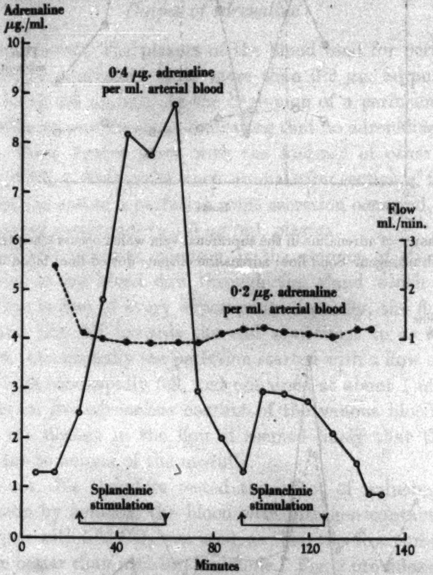

Fig. 4. The discharge of adrenaline produced by splanchnic stimulation. Solid line: adrenaline output; dotted line: blood flow.

Total adrenaline changes in system

Disappearance during anoxia. By taking the content of the right suprarenal medulla to represent that of the left at the start of the experiment, and by determining the output of adrenaline in the suprarenal vein, we discovered that, during anoxia, there was a loss of adrenaline from the system. For example, in an experiment in which potassium cyanide was injected, the right gland contained 2·32 mg./g. adrenaline. At the end of the experiment the weight of the left gland was found to be 0·534 g. and it then contained

0·47 mg./g. Assuming that its initial content was the same as that of the right, then

At the beginning it had 2320 × 0·534 =	1240 μg.
At the end it was found to contain	250 μg.
The loss was	990 μg.
The total excretion was	246 μg.
Hence there disappeared	744 μg.

Thus of the 1240 μg. adrenaline in the gland at the beginning 744 μg. was lost, which was 60%. A similar though smaller percentage loss was observed in the other experiments which are summarized in Table 2. We have no information concerning the fate of the missing adrenaline.

TABLE 2. Amounts of adrenaline μg.

Anoxia due to	Gland content Initial	Gland content Final	Total secretion	Net change	Percentage of initial
Nitrogen	2000	1250	235	−515	26
KCN	565	300	196	− 69	12
KCN	1240	250	246	−744	60
KCN	420	190	102	−128	30

TABLE 3. Changes during splanchnic stimulation (60 min.). Amount of adrenaline (μg.)

Adrenaline in arterial blood (μg./ml.)	Gland content Initial	Gland content Final	Total secretion	Net change	Change as percentage of initial adrenaline content
*0·3	2430	2320	495	+385	16
*0·4	625	400	288	+ 63	10
*0·45	1520	800	813	+ 93	6
0·45	875	682	323	+130	15
*0·6	1500	1068	486	+ 54	4
0·6	1350	1200	330	+180	13
0·7	3628	3020	664	+ 56	1·5
0·75	1071	1100	211	+240	23
1·25	1177	563	457	−157	14
1·5	705	530	223	+ 48	7
2·0	732	502	113	−117	16
2·5	1135	575	619	+ 59	6
7·0	1940	1200	310	−430	22
11·0	1856	1320	21	−515	28
16·0	1160	1400	−400	−160	14

Changes during splanchnic stimulation. In contrast to the experiments in which adrenaline secretion was produced by anoxia, stand the experiments in which secretion was produced by splanchnic stimulation applied for 1 hr. In these there was also a fall in the adrenaline content of the gland but this was accounted for by the secretion, and indeed there appeared to be a total gain. The details of these experiments are given in Table 3 marked by an asterisk; they are the only experiments in this series during which no adrenaline was added to the system at any time.

The presence of adrenaline in the suprarenal gland may be the result of the storage of adrenaline reaching the gland in the arterial blood. To examine this we added adrenaline to the perfusing blood in concentrations up to 16 μg./ml. and stimulated the splanchnic for 1 hr. In these experiments, too, we found that stimulation produced a total gain of adrenaline in the system (which might have been storage or synthesis) provided that the concentration of adrenaline in the arterial blood was less than 1·0 μg./ml. When the concentration was greater than this there was a net loss. The different results are shown in Table 3.

In view of the fact that the results in Tables 2 and 3 were obtained by biological estimations, and that each figure for the net change was subject to the cumulative error of several estimations, the conclusion might well be drawn that the Tables provide no evidence of any net loss or net gain whatever. This is particularly true since the net change, when expressed as a percentage of the adrenaline in the gland at the beginning, is, in Table 3, only once as high as 28%, and for the most part 15% or less. However, the net change for *each* experiment in Table 2, where there was anoxia, was a loss; on the other hand, the net change for *each* experiment in Table 3, with less than 1·0 μg. adrenaline in the arterial blood, was a gain, the mean figure being +150 μg.; finally, with more than 1·0 μg. adrenaline in the arterial blood, the net change was a loss in five out of seven experiments, the mean figure being −182 μg. These points suggest that some net loss and net gain actually occurred. The net gain, in the experiments in which the amount of adrenaline in the arterial blood was less than 1·0 μg./ml., was probably due to synthesis from unknown substances present either in the blood or in the gland, since the amount of adrenaline present in the arterial blood which might have been stored was so small. The loss of adrenaline, in the experiments in which the amount in the arterial blood was more than 1·0 μg./ml., might have been due to a restriction of the blood flow by the adrenaline thus producing anoxia. The evidence is partly in favour of this since the mean blood flow, at the end of the perfusions where there was less than 1·0 μg./ml. adrenaline in the arterial blood, was 0·74 ml./min., while, in those in which there was more adrenaline than this, it was 0·5 ml./min. The lowest terminal blood flow in any single perfusion was, however, the same in the two groups, namely, 0·4 ml./min.

The last experiment in Table 3 is one in which the secretion is recorded as negative. The amount of adrenaline in the venous blood was found to be less than that in the arterial blood during a considerable part of the splanchnic stimulation. This was the only experiment in which this was observed. It is possible that in this experiment adrenaline was stored.

Effect of arterial adrenaline on secretion

Relation of gland content to secretion. In experiments in which no adrenaline was added to the arterial blood the results supported the view that there was a relation between the amount of adrenaline in the gland and that liberated by splanchnic stimulation. We made no express investigation of this relation, and our results are not sufficiently numerous to enable us to claim that we have demonstrated that it exists. We can, however, quote three experiments in which the gland content was 0·86, 0·86 and 1·54 mg./g. and in which splanchnic stimulation liberated 1·9, 4·0 and 6·2 μg./min. respectively. With these, three other experiments can be compared in which the gland content was 1·73, 2·3 and 2·79 mg./g. and from which the discharge was 12·0, 8·0 and 7·8 μg./min. Thus a mean content of 1·09 mg./g. corresponded to a mean secretion of 4·0 μg./min., while a mean content of 2·3 mg./g. corresponded to a mean secretion of 9·3 μg./min.

In view of this evidence supporting what is inherently likely, we have expressed the result of splanchnic stimulation not only in μg./min. but also per mg. adrenaline present in the gland. An example of the calculation in one experiment is given in Table 4. The different samples were the successive portions of venous effluent which were collected, nos. 1 and 2 before, and nos. 3–7 during splanchnic stimulation for 60 min. The figure for secretion in the last column is the difference between the concentration of adrenaline in the venous and arterial plasma multiplied by the volume of the venous sample.

TABLE 4. Suprarenal perfusion. Adrenaline in plasma

Sample no.	Time (min.)	Arterial plasma (μg./ml.)	Venous plasma (μg./ml.)	Vol. of venous sample (ml.)	Secretion (μg.)
1	0	1·25	1·25	8·7	0
2	8	—	1·55	6·3	1·9
3	21	—	15·5	5·4	77·0
4	33	—	17·0	6·0	94·5
5	45	—	16·0	5·4	79·5
6	60	—	17·0	6·0	94·5
7	68	1·25	20·0	6·0	112·0

Secretion during splanchnic stimulation = 457·5 μg.
= 7·6 μg./min.
Adrenaline content of control gland = 2·13 mg./g.

Hence the secretion per min. per mg. of adrenaline content was 3·5 μg.

Effect of arterial adrenaline on secretion. Figures for adrenaline secretion per min. per mg. gland content in 17 perfusions have been plotted as ordinates in Fig. 5. The adrenaline concentrations in the arterial blood are plotted as abscissae. It was noticed that the addition of adrenaline had two effects. In those experiments in which the adrenaline concentration in the arterial blood was lowest, splanchnic stimulation caused the discharge of relatively small amounts of adrenaline from the gland. When the adrenaline content of the arterial blood was increased, the discharge due to splanchnic stimulation rose steeply. Beyond a certain optimum of adrenaline in the arterial blood, the discharge due to splanchnic stimulation declined and was finally abolished.

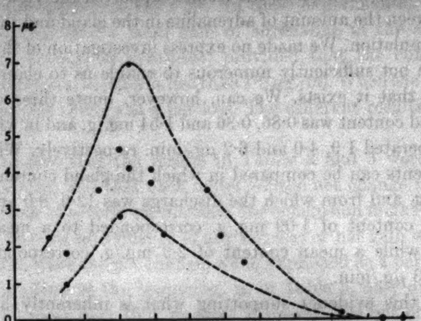

Fig. 5. Abscissae: adrenaline concentration in arterial blood on logarithmic scale. Ordinates: adrenaline secretion per min. per mg. gland content. As the adrenaline concentration in the arterial blood increased the secretion caused by splanchnic stimulation rose to a maximum and then declined.

The effect of a change in the arterial adrenaline in the course of one experiment is shown in Fig. 4. When the amount was 0·4 µg./ml. a greater secretion followed splanchnic stimulation than when it was 0·2 µg./ml.

DISCUSSION

It is well known that, in the body, asphyxia leads to secretion of adrenaline from the suprarenal glands. Houssay & Molinelli (1926) state that the effect is absent when the splanchnic nerves are divided. The results here described indicate that in the perfused suprarenal gland (with the splanchnic nerve cut) a period of anoxia leads to a discharge of adrenaline. This has been shown both by reducing the oxygen saturation of the blood and also by injecting potassium cyanide. Thus anoxia can also liberate adrenaline by peripheral action. It is possible that this action would not be exerted after degeneration of the splanchnic nerve, but it is difficult to see what part the ending of a severed nerve can play in it.

The observation that the result of splanchnic stimulation is affected by the concentration of adrenaline in the blood perfusing the gland is in keeping with our earlier finding that the transmission of impulses across the sympathetic synapse depends on the adrenaline concentration in the blood (Bülbring & Burn, 1942). It was shown that the addition of small amounts of adrenaline to the blood perfusing sympathetic ganglia augmented the peripheral vasoconstrictor response to preganglionic stimulation. The addition of further amounts of adrenaline depressed this response. These results were confirmed in the perfused cervical ganglion (Bülbring, 1944) in which again augmentation and depression of the postganglionic response could be produced by altering the concentration of adrenaline in the fluid perfusing the ganglion. Now, from experiments made to see if adrenaline could be stored in the suprarenal gland, we have obtained results which might have been expected from the known developmental relation of the suprarenal medulla to a sympathetic ganglion, and which therefore constitute a further confirmation of the earlier work.

Finally, it has been possible to follow the changes in the total amount of adrenaline in the perfusion system. In experiments involving anoxia, when adrenaline was discharged from the gland, the fall in the content of the gland was greater than was accounted for by this discharge. On the other hand, when adrenaline was discharged from the gland by splanchnic stimulation (provided the adrenaline content of the arterial blood was low), the loss from the gland was never greater than could be accounted for by the discharge. Indeed, the secretion exceeded the loss from the gland in each experiment, and, since the amount in the arterial blood was very small, this indicates that under favourable circumstances adrenaline may be synthesized in the perfused suprarenal.

There was one experiment only in which adrenaline appeared to have been stored. In this the arterial blood contained 16 μg./ml., and, during splanchnic stimulation, less adrenaline appeared in the venous effluent than was estimated in the arterial blood.

SUMMARY

1. The suprarenal gland of the dog was perfused with heparinized blood, and the output of adrenaline was estimated.

2. A fall in the oxygen supply to the gland, whether produced by a lowered arterial oxygen saturation or by deficient circulation, led to a rise in adrenaline output. The injection of potassium cyanide had the same effect. Consequently, the discharge of adrenaline in the body by anoxia may be a peripheral as well as a central action.

3. During anoxia there was a loss of adrenaline from the system, the loss in the gland exceeding the amount discharged by the vein.

4. A study was made of the amount of adrenaline liberated by splanchnic stimulation. This was found to depend on the amount of adrenaline present in the perfusing blood and possibly also on the amount initially present in the gland. When there was little adrenaline in the arterial blood, splanchnic stimulation caused the discharge of only small amounts of adrenaline from the gland. When the adrenaline content of the arterial blood was increased, the discharge due to splanchnic stimulation rose steeply. When, however, the

adrenaline content of the arterial blood was increased further beyond a certain optimum, the discharge due to splanchnic stimulation declined and was finally abolished.

5. Some evidence was obtained for the synthesis of adrenaline in the perfused gland.

This work was done by one of us (F. J. de E.) during the tenure of a scholarship from the Consejo Superior de Investigaciones Científicas, Madrid. We wish to express our thanks to Mr H. W. Ling for his very careful assistance with these experiments.

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EFFECT OF ULNAR NERVE BLOCK ON BLOOD FLOW IN THE REFLEXLY VASODILATED DIGIT

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While the presence of vasoconstrictor autonomic fibres leading to the skin has been demonstrated in mixed peripheral nerves, the existence of comparable vasodilator fibres is still in doubt. This report is an account of failure to demonstrate, physiologically, any such fibres present in the ulnar nerve at the level of the elbow and distributed to the fifth digit of healthy adult males.

DESIGN OF EXPERIMENTS

'*A*' *Series.* To determine whether ulnar nerve block reduces the degree of heat elimination:

(1) The simultaneous estimation of the heat elimination of the distal phalanges of the fifth digits of normal subjects in a state of 'full vasodilatation' (i.e. central abolition of vasoconstrictor tonus plus stimulation of any hypothetical vasodilator action) induced by prolonged immersion of the feet in water at 44° C. (Lewis & Pickering, 1931; Gibbon & Landis, 1932).

(2) The repetition of this procedure in the same subjects with one ulnar nerve blocked at the elbow by infiltration with 2% procaine and adrenaline.

'*B*' *Series.* To test the hypothesis that any nervous vasodilator impulses might only be demonstrable in vessels deprived of nervous vasoconstrictor tonus by central inhibition but held partially constricted by a local factor such as cold.

(1) The performance of experiments similar to '*A*' (1) with the difference that the fifth digit on one side was eliminating heat to a calorimeter containing water at 6–11° C.

(2) The repetition of this procedure in the same subjects with ulnar nerve block of the side subjected to the low ambient temperature.

METHODS

The foot baths used to induce vasodilatation were electrically heated, thermostatically controlled at 43·5° C. and stirred. A valuable safety precaution, additional to the normal 'third wire' earth, is the presence of a naked copper wire leading from the bath to a water pipe.

There are certain inherent limitations to the use of heat elimination as an index of blood flow, in particular the fact that actual blood flows can only be calculated if it is certain that the blood leaving the calorimeter has cooled to the calorimeter temperature. Mendlowitz (1941) made four observations of temperature in the dorsal vein of digits immersed in a calorimeter at about 31° C.; he found that the blood temperature averaged 0·6° C. higher than the calorimeter temperature. However, he used a needle thermocouple, the metal shaft of which was likely to allow a leakage of heat. We are in process of repeating these experiments with very fine copper-constantan junctions inserted into the vein by a needle which is afterwards withdrawn. In the experiments described in this paper, reliance is placed on the comparison of heat elimination between the two sides. In the '*A*' series these were maintained at identical states except for the controlled variable of ulnar block, and in the '*B*' series they were identical, except for a constant difference of calorimeter temperatures and ulnar block. When one side is at a low ambient temperature, although the rate is constant the magnitude of the difference of heat elimination is not regarded as bearing any simple relation to the magnitude of the difference in blood flow, as it is extremely unlikely that the blood leaving the cool calorimeter was cooled to 6–11° C. Furthermore, it has been shown by Bazett (1947) (whose observations became known to us after the completion of these experiments) that at a room temperature of 22° C. the blood in the radial artery was approximately 1° C. cooler than that in the brachial artery and that a considerable cooling of arterial blood was attributable to the cooler blood returning from the periphery in venae comites. In our experiments the influence of these factors is minimized by the room temperature being over 20° C., by the high rate of flow secured by reflex vasodilatation, and, when the cold calorimeter is used, by the fact that the cold blood from the immersed distal phalanx of the fifth digit constitutes only approximately 5% of the total blood flow of the hand according to the plethysmographic data of Wilkins, Doupe & Newman (1938) for the warm finger, and Freeman (1935) for the warm hand.

Blood temperature is assumed to be 37° C. in all experiments. As the subjects are already in a state of full vasodilatation by heat this value is unlikely to change during the course of the experiment. Individual differences are not likely to be greater than a degree or so and are equally operative on both sides.

Insulated copper calorimeters of approximately 100 c.c. capacity were used. These are similar to those described by Mendlowitz (1938) with the addition of (1) a small copper shelf 3·5 cm. from the top and a device to ensure that the water level is always 1 cm. from the top, so that a constant length (2·5 cm.) of the digit is always immersed; (2) a suitably perforated copper top deck to prevent splashing; and (3) an improved stirrer to ensure adequate mixing. Temperatures were indicated by Beckmann thermometers which, with the aid of magnifying eyepieces, permitted readings to 1/100° C.

Except in the 'cold' experiments initial temperatures of the calorimeter water were in the range of 27–33° C. and were the same on both sides. Temperature readings were made at half-minute intervals. In the 'cold' experiments the digit was immersed in unstirred ice water (under which conditions the temperature immediately surrounding the finger approximated to 6° C.) for 15 min. immediately before being placed in the calorimeter. Meanwhile, the subject was reflexly vasodilated and was, in fact, well on the way to dilatation before the finger was put in the calorimeter.

Calculation of results

In order to render results comparable it is necessary to express heat elimination as occurring over a uniform range. This can be achieved either by invariably performing experiments over this range or by extrapolation. The former method is laborious and, where the digit is being cooled by a cold calorimeter, impossible. If the logarithms of several observations of (37° C. – calorimeter temperature), plotted against time, fall along a straight line, then it may be assumed that the rate of heat elimination is constant and that the relationship of temperature (y) to time (x) can be expressed by the exponential formula $y = Ae^{-bx}$. Provided this criterion is satisfied the change of log $(37 - T)$ is constant per unit of time and the mean rise in temperature over any given range of 1° C. can be calculated.

Calorimeter above room temperature. The logarithms (to base 10) of (37° C. − calorimeter temperature) are plotted against time, and the steepest straight portion is taken as the period of maximum heat elimination (Fig. 1). The range 30–31° C. is taken as the test range and the time which would be taken to effect this rise of 1° C. is calculated by dividing the difference between the logarithms of 7 and 6 (37–30 and 37–31) by the logarithm change per min. during the period covered by the straight line. The reciprocal of this value is the mean rise in temperature in ° C./min. at maximum heat elimination in the test range.

The cooling of the calorimeter is observed immediately after the digit is withdrawn, and the results expressed as logarithms of calorimeter temperature minus the room temperature recorded close to the calorimeter. By logarithmic extrapolation the mean rate of fall over the test range of 31–30° C. is calculated, thus giving the rate of cooling.

The rate of cooling is then added to the rate of heating so as to obtain the rise in temperature which would have taken place in the perfectly insulated calorimeter. The volume of water in the calorimeter plus the thermal equivalent of the calorimeter and stirrer (the quite substantial thermal equivalent of the thermometer is neglected as it is a constant in all experiments) is then multiplied by the mean rise in ° C. thus obtaining the heat elimination in cal./min.

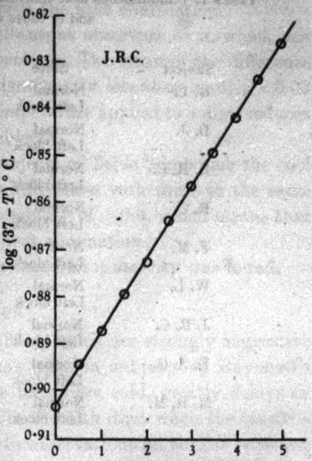

Fig. 1. Heating of calorimeter.

The surface of digit immersed is estimated by covering the distal 2·5 cm. with adhesive tape which is then removed, spread on a flat surface and this irregular area measured with an Amsler's Planimeter.

The results are finally expressed as cal./sq. cm./min.

Calorimeter below room temperature. In these experiments the calorimeter receives heat from both the digit and the room. The method devised for dealing with this situation is to express the total heating as a function of the range 9–10° C. and to express the heating due to the ambient temperature as a function of the same range. The difference between these two functions represents the heating that would have taken place in a perfect calorimeter if the sole source of heat had been the digit. This figure is finally expressed as a function of the range 30–31° C. so that it is comparable with heat elimination performed at calorimeter temperatures above room temperature. These observations and calculations have been greatly simplified by the use of proformata. With the aid of a hand-driven calculating machine it is possible to complete the calculations for a measurement of bilateral simultaneous heat elimination in approximately 40 min.

RESULTS

Table 1 gives the results of simultaneous heat elimination from the fifth digits of nine normal subjects, both without and with the left ulnar nerve blocked; the last column expresses the extent to which the left side differs from the right. A statistical comparison was made of the group of differences in the normal state and the corresponding group when the left ulnar nerve was

TABLE 1. Simultaneous heat eliminations in cal./sq.cm./min. of normal fifth digits and of those with the ulnar nerve blocked

Subject	State	Fifth right digit	Fifth left digit	Difference
R. L.	Normal	1·76	1·84	+0·08
	Left block	1·72	1·74	+0·02
D. J.	Normal	2·02	2·39	+0·37
	Left block	2·19	2·50	+0·31
E. H. C.	Normal	1·77	1·89	+0·12
	Left block	1·43	1·93	+0·50
R. C.	Normal	2·09	1·99	−0·10
	Left block	2·60	2·57	−0·03
F. M.	Normal	2·50	2·18	−0·32
	Left block	2·14	1·99	−0·15
W. L.	Normal	1·83	1·94	+0·11
	Left block	1·97	2·02	+0·05
J. R. C.	Normal	2·06	2·14	+0·08
	Left block	2·24	2·02	−0·22
D. A. G.	Normal	1·97	1·70	−0·27
	Left block	1·97	1·95	−0·02
M. R. M.	Normal	2·40	1·89	−0·51
	Left block	2·32	1·99	−0·33

TABLE 2. Simultaneous heat eliminations in cal./sq.cm./min. of fifth digits one side being at a low ambient temperature

Subject	'Cold' fifth digit	'Warm' fifth digit	Difference
R. F. D.	1·76	2·31	−0·55
J. R. C.	1·97	1·89	+0·08
R. L.	2·13	2·46	−0·33
F. L. R.	1·69	2·21	−0·52
H. G. C.	1·33	1·71	−0·38
R. F. F.	2·03	2·51	−0·48
W. M. A.	1·97	1·92	+0·05
P. A. G.	1·82	2·83	−1·01

TABLE 3. Simultaneous heat eliminations in cal./sq.cm./min. of fifth digits at a low ambient temperature, one digit being normal and the other ('cold') with the ulnar nerve blocked

Subject	State	'Cold' fifth digit	'Warm' fifth digit	Difference
J. R. C.	Normal	1·97	1·89	+0·08
	Ulnar block	1·63	2·16	−0·53
R. L.	Normal	2·13	2·46	−0·33
	Ulnar block	1·43	1·84	−0·41
F. L. R.	Normal	1·69	2·21	−0·52
	Ulnar block	1·43	1·89	−0.46
H. G. C.	Normal	1·33	1·71	−0·38
	Ulnar block	1·81	1·98	−0·17
R. F. F.	Normal	2·03	2·51	−0·48
	Ulnar block	2·07	2·14	−0·07
W. M. A.	Normal	1·97	1·92	+0·05
	Ulnar block	1·92	1·76	+0·16

blocked (Fisher, 1944); the result was $P < 0{\cdot}7$ and $> 0{\cdot}6$, that is the groups were homogeneous, ulnar block not having altered the heat elimination.

Table 2 gives the result of similar simultaneous observations in which one side was subjected to a low ambient temperature. The mean of the difference between the two sides was found to be significantly less than zero, $P < 0{\cdot}02$ and $> 0{\cdot}01$; that is to say, a low ambient temperature applied to a digit reduces heat elimination in reflexly vasodilated subjects.

Table 3 gives the results in six of the subjects of Table 2 in which the cool side was 'blocked'. A comparison of the differences with those in the same subjects without ulnar block yielded a probability of $> 0{\cdot}9$, which means that blocking has not significantly altered the heat elimination.

In brief, no evidence of specific nervous vasodilator activity was found.

DISCUSSION

The observations of Lewis & Pickering (1931), which are strongly suggestive of the presence of vasodilator fibres, show that in subjects of Raynaud's disease ulnar block, carried out when the hands are cold, greatly delays or even prevents the onset of vasodilatation in the fifth digit when the trunk is strongly heated. This is in sharp contrast to observations on similar patients, who possess intact innervation and in whom strong central heating secures release of digital vasoconstriction, and also to those on cool normal subjects whose digits can be caused to vasodilate by ulnar block. These authors subjected six cats to unilateral stellate ganglionectomy and found that heating the trunk resulted in a relatively greater rise of temperature of the normally innervated ear and of the fore-paw pad in four of the animals, a rise to the same level in one, and to a less degree in one. These results while not conclusive were regarded as suggestive of the presence of vasodilator fibres in the cat. In man, the same workers compared the skin temperatures (by thermocouples) of the right and left fifth digits of warm normal individuals with ulnar block of one side; no significant difference was found. They concluded that 'a failure to display existing vasodilator sympathetic fibres in normal men and cats is really not surprising; after vasoconstrictor tone has been lost the temperature of the part supplied rises so close to the temperature of the blood entering it, that the accession of vasodilator impulses has little chance, perhaps none, of displaying itself in a further rise of temperature'. The calorimetric method overcomes to a large extent this limitation.

Fatheree & Allen (1938), using thermocouples, have confirmed these observations of Lewis & Pickering in cases of Raynaud's disease but have failed to demonstrate the presence of sympathetic vasodilator fibres in the extremities of normal persons. They performed ulnar nerve block in an environmental temperature of 0° C. which might have overcome the vasodilator effects of blocking the central vasoconstrictor impulses. Vasodilatation still occurred.

Sarnoff & Simeone (1947) (whose paper became available after our experiments were concluded) found that block of the nerve supply to both fingers and toes did not diminish the high level of skin temperature due to reflex vasodilatation.

This failure to demonstrate vasodilator fibres to the fifth digit in normal subjects may be reversed by a design of experiment which has so far eluded us, but it indicates that, if they do exist, their role in purely physiological adaptation is insignificant. On the other hand, the quoted experiments on Raynaud's disease seem to point conclusively to their existence. The explanation of this discrepancy may lie in the fact that such a vasodilator mechanism is negligible in healthy individuals but becomes developed in individuals who are constantly subjected to digital ischaemia determined by local vascular hypersensitivity to cold. Even if this be so, we agree with Sarnoff & Simeone that it should not be taken as evidence against performing sympathectomy in cases of Raynaud's disease.

In conclusion it should be emphasized that this report deals only with the possibility of vasodilator fibres to digits. The observations of Grant & Holling (1938) afford strong evidence of the existence of vasodilator fibres distributed to the skin of the forearm and the investigations of Barcroft & Edholm (1945) provide physiological proof of vasodilator fibres to muscle.

SUMMARY

1. Ulnar nerve block does not significantly alter the blood flow (as estimated by calorimetry) of the fifth digit in normal human subjects in a condition of reflex vasodilatation.

2. Local cooling of the fifth digit in reflexly vasodilated subjects significantly lowers the blood flow, the degree of which is not further altered by ulnar block.

We wish to express gratitude to Prof. Henry Barcroft for the design of the heating-stirrer unit, to our Senior Technician, A. C. Pincock, for his skilled assistance and to the volunteers for their willing service.

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ASCORBIC ACID IN ADRENAL BLOOD

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Sayers, Sayers, Liang & Long (1945, 1946) and Long & Fry (1945) have shown that a loss of ascorbic acid from the rat's adrenal cortex occurs during stress and after injection of adrenaline or of corticotrophic hormone. These conditions are presumably associated with increased cortical secretion. The role of the vitamin in the secretory process is not known, but the explanation has been sought along two lines: either the vitamin may be required for the metabolic processes leading to hormone synthesis, or it may form an actual part of the hormone molecule released into the blood. The latter possibility was suggested by the observation of Zwemer & Lowenstein (1940) and Lowenstein & Zwemer (1946) who isolated from adrenal glands a glycoside-like substance consisting of ascorbic acid and a ketonic steroid. It seemed of interest, as suggested to the author by C. N. H. Long, to try to follow the fate of the ascorbic acid which disappears from the secreting cortex and to see whether the vitamin could be found, either in a free or in a bound state, in the venous outflow of the adrenal. Adrenal blood should be a good source for the hypothetical glycoside, since earlier experiments (Vogt, 1943) have shown that such blood, collected from anaesthetized dogs or cats during an abdominal operation, invariably contains more cortical hormone than can be extracted from the same weight of glandular tissue. If consistent changes in the ascorbic acid content of adrenal blood accompanied the secretory process, ascorbic acid estimations could be used as a convenient method of obtaining information on cortical activity.

METHODS

Operative procedures

Adrenal venous blood was obtained in the manner previously described (Vogt, 1943) from eviscerated cats or dogs anaesthetized with chloralose. Bound vitamin C was estimated in the adrenal blood of cats. The effluents of both adrenals were collected, and control blood taken from the carotid artery or the heart. Infusions of 0·9% NaCl solution or blood were required to keep up a sufficient blood flow. Free vitamin C was estimated in the plasma of dogs, since their greater blood volume allowed extensive bleeding without intravenous infusions which might alter the ascorbic acid content of the plasma. As the adrenal blood was collected from the left gland only, the right adrenal was extirpated in order to avoid changes in the ascorbic acid content of the arterial blood which might arise from the activity of the right gland during the experiment. For the same reason, no effluent from the left adrenal was returned into the general circulation once the collection of blood samples had started. The arterial samples were collected from a carotid artery, simultaneously with the adrenal blood.

Chemical methods

Estimation of ascorbic acid was carried out in plasma and in plasma extracts by the method of Roe & Kuether (1943) which determines both ascorbic and dehydro-ascorbic acid. No special precautions were therefore required to prevent the formation of dehydro-ascorbic acid. The blood was collected in ice-cooled tubes and centrifuged. The plasma was analysed immediately.

Extracts were prepared by shaking the plasma with an equal volume of ethylene dichloride, separating the layers by centrifuging, extracting the supernatant fluid a second time with ethylene dichloride, centrifuging again and filtering the combined layers of organic solvent into a distilling flask. The ethylene dichloride was distilled off in vacuo at a bath temperature of between 50 and 60° C. Before the residue was dry, 10 c.c. absolute alcohol were added and the remainder of the ethylene dichloride distilled off. Similarly, a few c.c. of water were poured into the flask before the alcohol had evaporated completely. In this way, any steroids present in the extract were transferred from the organic solvent into water while remaining in solution all the time. The watery extract was acidified with one-tenth of its volume of N-HCl and divided into two halves, one of which was incubated for 1 hr. at 38° C. while the other was kept in the refrigerator. Before the oxidation of the ascorbic acid by charcoal according to the method of Roe & Kuether, trichloracetic acid had to be added to the solutions, as in its absence the oxidation of ascorbic acid and the osazone formation with dinitrophenylhydrazine remained incomplete. Recovery experiments in which ascorbic acid was mixed with extracts which were subsequently incubated with HCl gave satisfactory yields, provided that trichloracetic acid (3·5–4%) was added before the treatment with charcoal.

Direct estimations of plasma ascorbic acid were carried out after precipitation of the proteins with a small volume (4 c.c. for every 10 c.c. plasma) of 12% trichloracetic acid, thus avoiding undue dilution of the vitamin.

RESULTS

Plasma extracts. Previous (unpublished) experiments had shown that ethylene dichloride extracts of adrenal plasma, prepared as described above, contain biologically active cortical hormone, and that similarly prepared extracts of arterial blood have no such activity. Satisfactory agreement was obtained in control experiments in which the activity of adrenal plasma was compared with that of its extract, or in which commercial cortical preparations were added to arterial plasma and the recovery tested biologically after having proceeded with the extraction. It should, however, be emphasized that the biological assay is not sufficiently accurate to give information as to whether some loss occurs in the process, but it does show that such extracts contain at least a considerable fraction of the active steroids present in plasma. If an appreciable proportion of these steroids contain ascorbic acid in combination, the compound should presumably be found in the ethylene dichloride extract.

In the first experiment, the final volume of extract was made equal to the original volume of plasma extracted, and in three further experiments it was reduced to one-half. No ascorbic acid was detected in any of these extracts, either before or after acid hydrolysis. Since it is quite easy to detect 0·5 μg. vitamin C per c.c. solution by Roe & Kuether's method, the original plasma contained less than 0·25 μg. ascorbic acid per c.c. as ethylene dichloride-soluble glycoside. This means either that the adrenal effluent does not contain a large fraction of its cortical steroids in the form of a glycoside, or that such a compound is destroyed by shaking the blood with the organic solvent, or that it is not hydrolysed by 0·1N-HCl at 38° C.

TABLE 1. Changes in plasma ascorbic acid observed in blood perfusing the adrenal

1	2	3	4	5	6	7	8
		Ascorbic acid (μg./c.c.)				Loss or gain of ascorbic acid on passage of plasma through gland	
No. of dog	No. of sample	Arterial plasma	Suprarenal plasma	Difference	Flow (c.c. plasma/min.)	(μg./min.)	(μg./min./kg.)
1	1	10·6	8·8	−1·8	0·11	−0·21	−0·018
2	1	7·6	6·5	−1·1	1·25	−1·38	−0·21
	2	8·0	7·1	−0·9	1·28	−1·15	−0·17
3	1	4·3	3·9	−0·4	1·43	−0·57	−0·11
	2	4·2	4·75	+0·55	1·14	+0·63	+0·13*
4	1	6·7	7·1	+0·4	0·64	+0·26	+0·035
	2	7·0	8·4	+1·4	0·52	+0·73	+0·10
	3	8·3	9·8	+1·5	0·35	+0·52	+0·07
5	1	11·5	12·3	+0·8	0·34	+0·27	+0·04
6	1	6·7	7·3	+0·6	0·68	+0·41	+0·05
	2	7·6	8·3	+0·7	0·55	+0·38	+0·04†
	3	11·2	9·3	−1·9	0·45	−0·86	−0·10*

* In these experiments the dog died during collection of the last sample.
† Stimulation of the splanchnic nerves.

Direct estimations in plasma. The results are seen in Table 1. There were small differences in the ascorbic acid level of arterial plasma and adrenal effluent, but as the blood passed through the gland, gains and losses occurred about equally often. Since, in such experiments, the rate of secretion of cortical hormone per unit of time remains constant over long periods in spite of variations in blood flow (Vogt, 1944), any correlation between secretion and plasma ascorbic acid should be evident in columns 7 and 8. In column 7 the amounts of ascorbic acid gained or lost are calculated per min., and in column 8 the changes are given per min. and per kg. body weight. All the figures, however, are very variable, and positive and negative values alternate even within one experiment.

A relation between the occurrence of vitamin C in adrenal effluent and the secretion of adrenaline by the medulla has been suggested by Heard & Welch (1935) as a result of observations on perfusions of ox glands with Locke's solution. In Exp. 6, therefore, the left splanchnic nerves were cut and the peripheral ends stimulated during collection of the second pair of blood samples.

Comparison of the results before and during stimulation shows that this procedure did not affect the small rise in vitamin C found in the adrenal effluent.

DISCUSSION

If the whole of the cortical steroids present in adrenal plasma were combined with ascorbic acid, the quantity of bound vitamin to be expected might be assessed from the known order of magnitude of the steroid secretion. Under conditions identical with those of the present experiments, this was found to amount, per min. per kg., to the equivalent of 0·6 g. cortical tissue (Vogt, 1943) or to about 2 μg. of Kendall's compound E (17-hydroxy-11-dehydro-corticosterone). From the blood flow in the present experiments on cats we can calculate that each c.c. of plasma was likely to contain between 12 and 31 μg. of compound E and therefore about half that amount of bound ascorbic acid. However, less than 0·25 μg. of ascorbic acid per c.c. plasma were found in extracts of suprarenal blood in which at least the greater part of the cortical hormone must have been present. Thus it is evident that, if any glycoside exists in such blood, it represents either a small fraction which is not soluble in ethylene dichloride, or it is so labile that shaking with the organic solvent destroys it, or so stable that incubation with 0·1 N-HCl fails to hydrolyse it. The latter alternatives are unlikely since they disagree with the observations of Lowenstein & Zwemer (1946) that their glycoside can be extracted with chloroform and is split by 'mild hydrolysis'.

Since there was no consistent difference between the ascorbic acid content of the blood entering and leaving the adrenal gland, the ascorbic acid content in the adrenal effluent cannot be used as an index of cortical secretion.

Finally, stimulation of the splanchnic nerves did not affect the vitamin C content of adrenal blood. There is thus no evidence in favour of Heard & Welch's suggestion of a relation between secretion of adrenaline by the medulla and ascorbic acid content of the blood leaving the adrenal. Perhaps this discrepancy is a result of the different behaviour of a gland irrigated with Locke's solution and one perfused with blood.

SUMMARY

1. Ethylene dichloride extracts of plasma obtained from adrenal blood of cats do not give any reaction for ascorbic acid either before or after incubation with 0·1 N-HCl for 1 hr. at 38° C. Since such extracts contain at least a large fraction of the cortical steroids, it is unlikely that much cortical hormone in adrenal blood is linked to ascorbic acid.

2. The ascorbic acid level of plasma prepared from adrenal blood of dogs may be either a little above or below that of arterial plasma taken at the same time. Even during a single experiment, and under conditions associated with a steady cortical secretion, the differences may be in opposite directions. There is thus no correlation between the contents of ascorbic acid and of cortical hormone in the plasma obtained from the adrenal blood.

3. No relationship has been found between adrenaline secretion and ascorbic acid content of adrenal effluent.

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THE EFFECTS OF SUCROSE UPON GRUEL FRACTIONAL TEST MEALS

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From Guy's Hospital Medical School

ERRATA

Vol. 106.

P. 72, fig. 5. *The lower voltage calibration should read* 0·2 mV. *instead of* 0·5 mV.

P. 37 *P*, line 6. *Delete the phrase* '2a giving an improved statistical treatment'; *and also the appendix to* Table 1 *beginning* 'Further statistical treatment'.

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resting juice had been withdrawn, took a pint of warm gruel to which had been added weighed amounts of sucrose. In all cases the gruel was made with 45 g. rolled oats in 2 pints of water, the mixture being slowly boiled till the volume was halved. After taking the gruel small samples of stomach contents were withdrawn every 15 min. until one was obtained which did not give a blue colour with iodine. The interval of time up to the last sample giving a colour was taken as the emptying time. The subjects did not restrict their activity during the test meal. A few experiments were rejected as unsatisfactory as, owing to gastric distress, it was not possible to obtain samples or the subjects were nauseated. Each sample obtained was titrated with 0·1 N-NaOH, with phenolphthalein as an indicator, to determine the 'total acidity'. The meals for each subject, which were spaced over periods as long as 18 months, were performed under the close personal supervision of the authors.

RESULTS

Ten subjects were used in a total of seventy-four test meals. Of these one has been rejected because of nausea. Further, all the eleven results from one subject whose stomach responded quite irregularly both to control meals and meals containing sugar have been omitted. These discrepancies appear to have been partly due to irregular motor activity induced by the Ryle's tube. This leaves for consideration a total of sixty-two test meals on nine subjects.

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THE EFFECTS OF SUCROSE UPON GRUEL FRACTIONAL TEST MEALS

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Whilst it is widely recognized that the addition of glucose to gastric test meals reduces the acidity of the gastric contents and delays the emptying of the stomach, it does not appear to be known whether or not sucrose produces similar effects in concentrations which are palatable. However, sucrose is the usual flavouring agent in gruel test meals and instructions not infrequently include the expression 'sugar may be added to taste'. It seemed, therefore, of some practical interest to determine whether or not variations in the concentration of sucrose in a meal could influence results to a degree significant for clinical interpretation.

METHODS

The subjects, who were healthy men and women working in the laboratory, reported in the morning having fasted since the previous evening. They swallowed a Ryle's tube, and, after the resting juice had been withdrawn, took a pint of warm gruel to which had been added weighed amounts of sucrose. In all cases the gruel was made with 45 g. rolled oats in 2 pints of water, the mixture being slowly boiled till the volume was halved. After taking the gruel small samples of stomach contents were withdrawn every 15 min. until one was obtained which did not give a blue colour with iodine. The interval of time up to the last sample giving a colour was taken as the emptying time. The subjects did not restrict their activity during the test meal. A few experiments were rejected as unsatisfactory as, owing to gastric distress, it was not possible to obtain samples or the subjects were nauseated. Each sample obtained was titrated with 0·1 N-NaOH, with phenolphthalein as an indicator, to determine the 'total acidity'. The meals for each subject, which were spaced over periods as long as 18 months, were performed under the close personal supervision of the authors.

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The experimental results for meals containing no sugar have been grouped for each subject. The mean value of each grouping has been calculated to provide a basis for comparison and the results for any test meal have been expressed as a percentage of the basic mean of the subject concerned.

Table 1 shows the influence of sucrose on the emptying time of the stomach. The differences between the mean of the controls and the mean of the tests have been calculated, together with the standard errors of these differences. If sugar has no influence on the experimental results one may expect the value of these differences to average zero. *P* is a measure of the significance of these results, and values of 0·05 or less are accepted as indicating a statistically significant difference. When 100 g. sucrose were added to the meal the mean delay was 22%; this figure is certainly significant, and 100 g. of sucrose is not beyond the range of choice for normal individuals. Occasional subjects found that addition of 100 g. sucrose made the meal slightly nauseating; in one such subject a 75% delay was observed. On the other hand, with addition of 12–25 g. sucrose to the meal the stomach emptied significantly more rapidly than with the control meal.

TABLE 1. The effect of variation in the concentration of sucrose on the 'emptying time' of fractional test meals

Amount of sugar in meal (g.)	No. of tests	No. of subjects	Mean emptying time as % of control	Standard error of mean	Diff. between mean of tests and controls	Standard error of diff.	*P* derived from Student's '*t*'
0	21	9	100·00	2·75	—	—	—
12–25	9	4	85·22	4·72	−14·77	5·47	0·01
35–65	8	4	109·37	6·11	+ 9·37	6·70	0·2
100	17	9	122·71	5·27	+22·7	5·94	0·001

Experimental results from control meals containing no sucrose are grouped for each subject. The results for any test meal are expressed as a percentage of the mean value for the subject concerned.

In Table 2 is shown the influence of variations in concentration of sucrose on the mean level of 'total acidity' during the first hour after the taking of the meal, excluding the values for the resting juices. It may be seen that the concentration of acid is uniformly depressed by even small amounts of sucrose and that this depression is statistically significant for the groups with concentrations of 35 g. or more per pint of test meal.

TABLE 2. The effect of variation in the concentration of sucrose on the mean 'total acid' in the first hour of fractional test meals

Amount of sugar in meal (g.)	No. of tests	No. of subjects	Mean total acid during 1st hour as % of control	Standard error of mean	Diff. between mean of tests and controls	Standard error of diff.	*P* derived from Student's '*t*'
0	21	9	100·00	3·82	—	—	—
12–25	9	4	92·00	11·22	− 8·00	11·87	0·5
35–65	9	4	63·11	7·03	−36·89	8·01	0·001
100	17	9	47·29	3·67	−52·70	5·31	<0·001

Table 3. The effect of variation in the concentration of sucrose on the mean 'total acid' of fractional test meals excluding the first hour

Amount of sugar in meal (g.)	No. of tests	No. of subjects	Mean total acid as % of control	Standard error of mean	Diff. between mean of tests and controls	Standard error of diff.	*P* derived from Student's '*t*'
0	20	9	100·00	5·72	—	—	—
			Group A				
12–25	3	1	114·00	10·10	+14·00	11·48	0·3
35–65	1	1	125·00	—	—	—	—
100	7	4	118·57	6·75	+18·57	8·86	0·05
			Group B				
12–25	5	3	81·60	9·11	−18·40	10·71	0·1
35–65	7	4	75·86	7·04	−24·14	9·05	0·02
100	9	5	59·00	4·10	−41·00	7·04	<0·001

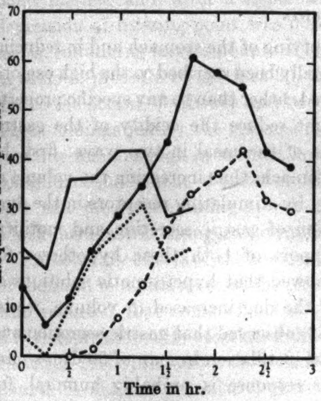

Fig. 1.

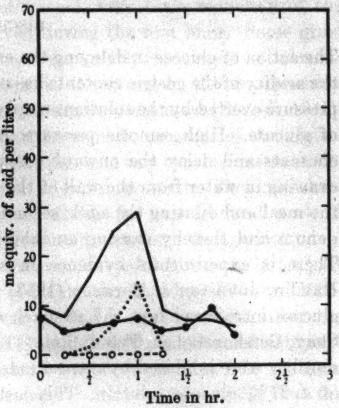

Fig. 2.

Fig. 1. The effect of adding 100 g. of sucrose to a test meal for a subject of group A. No sucrose: —— total acid; ····· free acid. 100 g. sucrose: ●—● total acid; ○- - ○ free acid.

Fig. 2. The effect of adding 35 g. of sucrose to a test meal for a subject of group B. Control meal, no sucrose: —— total acid; ····· free acid. With 35 g. sucrose: ●—● total acid; ○- - ○ free acid.

Table 3 shows the effect of additions of sucrose on the mean 'total acidity' excluding the first hour during which uniform depression has already been demonstrated. The nine subjects fall into two groups, A and B. The four subjects in group A show an increase in the mean total acidity of the meal following the first hour with 100 g. of sucrose, whilst the five subjects in group B show a significant decrease in acidity with amounts of sugar greater than 35 g. Fig. 1 shows the results of two typical test meals for a subject of group A, one meal containing no sucrose and the other containing 100 g. The reduction in acidity during the first hour followed by a high acidity, as compared with the control, may be seen. There is slight delay in the emptying of the stomach. Fig. 2 shows the results of two test meals, one containing no sucrose and the other containing 35 g., for a subject of group B. The acidity of the gastric contents was so reduced that no free acid was found. The results shown are typical of five experiments on the first subject and of fourteen experiments on the second subject. The difference between the means shown for the two groups in Table 3 for the secretory responses to 100 g. sucrose is 59·57; the standard error of this difference is 7·9. Using Student's '*t*' the value of P is $<0{\cdot}001$. Thus there appears to be a very significant difference between the two groups. Further, we have seen no evidence of subjects passing from one group to the other in the 18 months during which some of them were observed.

DISCUSSION

The action of glucose in delaying the emptying of the stomach and in reducing the acidity of the gastric contents has usually been ascribed to the high osmotic pressure exerted by the solutions employed rather than to any specific property of glucose. High osmotic pressure might reduce the acidity of the gastric contents and delay the onward passage of the meal in two ways: first, by drawing in water from the wall of the stomach, thus increasing the volume of the meal and diluting the acid; secondly, by stimulating receptors in the duodenum and thereby causing an inhibition of gastric secretion and motility. There is experimental evidence in support of both these hypotheses for Ravdin, Johnston & Morrison (1933) showed that hyperosmotic solutions of glucose introduced into the stomach of the dog increased in volume, whilst Shay, Gershon-Cohen, Fels & Siplet (1942) observed that gastric secretion and motility were inhibited by intra-duodenal instillation in man of 30 drops/min. of a 27% glucose solution. This latter response is probably humoral, for Quigley & Phelps (1934) showed that a solution of glucose given into the alimentary tract inhibited the movements of an auto-transplanted pouch whilst glucose injected intravenously had very little effect.

The hastened emptying observed with concentrations of sucrose between 12·5 and 25 g. per pint has not been recorded by other investigators though Spencer, Meyer, Rehfuss & Hawk (1916) described a similar acceleration with a 1% solution of $NaHCO_3$. Apperly (1926) has suggested that 'the stomach retains its contents until a suitable osmotic pressure, roughly isotonic with blood, is reached'. Although there seems to be no justification for the conclusion about retention there is evidence that a stomach empties more quickly when the ingested meal is at an optimum concentration and it is possible that our gruel meal with 12·5–25 g. of sucrose added is near that optimum. There is no reason why the osmotic pressure at the optimum point should be isotonic with blood, as far as osmotic effects are concerned. The deciding factor must be the effective osmotic pressure exerted at the mucosal surface and in this laboratory we have many measurements which show that the final depression of freezing-point of gastric contents following test meals is about 0·3° C. which is half that of blood plasma.

If the results with added sucrose are to be considered as due to a similar osmotic effect, the uniform reduction of the acidity of the gastric contents during the first hour might be expected for two reasons. First, any osmotic effects of a hyperosmotic meal would presumably be maximal at the beginning of the meal; and secondly, the cephalic phase of gastric secretion has been observed to be particularly sensitive to inhibition by hyperosmotic glucose solutions instilled into the duodenum (Shay *et al.* 1942). In a number of cases we have measured the osmotic pressure of the gastric contents by the freezing-point method and, after a hyperosmotic meal, very little reduction of the depression of freezing-point was observed during the first hour. Some gruel meals, containing large amounts of sucrose, apparently underwent an increase in osmotic pressure in the first 30–45 min. in the stomach: this was possibly due to the action of salivary amylase on the starch of the meal (Beazell, 1941). These findings justify the statement that the osmotic pressure of the gastric contents remains high in the first hour, but preclude any conclusions as to the consequent movement of water or dilution of gastric contents. They also show that test meals containing starch are unsuitable for experimental work in which intragastric changes in osmotic pressure are under investigation.

The contrary effects of the sucrose in different subjects during the latter part of the meal is reminiscent of the effect of fat demonstrated by Roberts (1925) who reported that some of his subjects showed a hypersecretion after the initial depression. He correlated this phenomenon with the work of Babkin & Sawitsch (1908) in which they demonstrated that soaps given into the duodenum in animals stimulated gastric secretion. It seemed possible that the increased acidity might be due to increased secretion induced by products of the proteolytic digestion of the gruel. If this were so those subjects showing minimal depression of acidity during the first hour might perhaps be expected to show maximal secretion during the remainder of the meal. The results of these experiments are insufficient to test this correlation. Since the total chlorides have not been estimated it is impossible to express any opinion as to the part played by duodenal regurgitation in this phenomenon. It is remarkable that the subjects fall so clearly into two groups although an examination of nine subjects does not warrant the assumption that no intermediate groups may subsequently be found.

The inhibiting effect of the stronger solution of sucrose on the emptying of the stomach might be expected from the work of Quigley & Phelps (1934) who showed that strong solutions of sucrose given intra-duodenally in dogs inhibited gastric motility even in the completely denervated stomach. It is in harmony with the work of Van Liere, Northup & Stickney (1946), who studied the influence of glucose added to barium meals in man and noted a significant increase in the emptying time when amounts in excess of 25 g. of glucose were added to 200 ml. of barium mixture.

The value of the test meal in the diagnosis of peptic ulcer and dyspepsia is being increasingly challenged (Alvarez, 1943) but the test is still considered to be useful in the diagnosis of neurological disorders where the presence of free HCl may be of considerable significance. Fig. 2 shows that the addition to the test meal of only 35 g. of sucrose, which is well within the limits of choice, may completely mask the capacity of the stomach to reduce the pH of its contents to 3·5. No clinician would accept such a negative result as significant, but, nevertheless, such a patient would be subjected to a further test with histamine which might have been avoided if sucrose had been excluded from the meal.

SUMMARY

1. The addition of sucrose to gruel fractional test meals reduces the acidity of the gastric contents during the first hour. Subsequently, it either depresses the acidity or increases it, but the response is uniform for each individual tested.

2. The addition of 12–25 g. of sucrose to the test meal significantly reduces the 'emptying time', but addition of 100 g. of sucrose significantly increases the 'emptying time'.

We are indebted to our subjects for their endurance.

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THE INFLUENCE OF THYROXINE AND ADRENAL CORTICAL EXTRACT ON THE OXYGEN CONSUMPTION OF ADRENALECTOMIZED RATS

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Considerable disagreement exists as to whether the cortical hormone exerts an influence on the calorigenesis of normal animals or not. According to Koehler & Hastings (1929), the administration of cortical extract to dogs and mice increases the oxygen consumption, while Franke (1932) has observed that subcutaneous injections of cortical hormone lead to a decrease of metabolism in dogs. Harrop, Pfiffner, Weinstein & Swingle (1931) have not found a change in the caloric output of normal animals when cortical hormones were administered. Neither was there any influence of cortical substances on basal metabolism of man (Hitchcock, Grubbs & Hartman, 1938). In contrast to these conflicting results, those obtained by administration of cortical hormones in adrenal insufficiency are very consistent, and in these cases considerable increase of oxidation has regularly been observed (Brownell & Hartman, 1941; Hartman, Brownell & Crosby, 1931; Swingle & Pfiffner, 1932).

The removal of the adrenal glands causes a decline of calorigenesis (v. Arvay, 1929; Aub, Bright & Uridil, 1922; Barlow, 1924; Brownell & Hartman, 1941; Carr & Connor, 1933; Demaria-Massey, 1927; Swingle & Pfiffner, 1932; Webster, Pfiffner & Swingle, 1932). Earlier findings which seemed to show that this operation leads to a rise of respiratory metabolism (Marine & Baumann, 1921, 1922*a*, *b*) can be explained by the assumption that the adrenalectomy was not complete (Webster *et al.* 1932). Marine & Baumann (1921, 1922*a*, *b*) believe that the relatively high metabolic rate of adrenalectomized rabbits is due to a hyperfunction of the thyroid gland as they did not observe an increase of calorigenesis in thyroidectomized animals. Demaria-Massey (1927) reported a lowering of respiratory metabolism of rats after thyroidectomy as well as after adrenalectomy and a pronounced decline of caloric output after removal of both glands.

The calorigenic action of the cortical hormone in adrenal insufficiency apparently does not exert itself through an increase of the thyroid function, as it appears also in thyroidectomized animals (Webster *et al.* 1932). On the other hand, it has been observed that relatively small doses of thyroid hormone cause a severe crisis in animals (Carr & Connor, 1933; Kendall, 1940; Koelsche & Kendall, 1935) and in patients (Anderson & Lyall, 1937; Harrop, Weinstein, Soffer & Trescher, 1933; Means, Hertz & Lerman, 1940) with adrenal insufficiency, but little is known about the calorigenic effect of thyroid hormone in absence of the adrenal glands. While the experiments reported here were in progress there appeared a paper by Doetsch, Verzar & Wirz (1945), which provides evidence that administration of thyroxine to adrenalectomized cats, kept alive with small doses of desoxycorticosterone (D.O.C.), exerts but little effect on respiratory metabolism. The authors were able to elicit a normal calorigenic response to thyroxine by giving simultaneously large doses of D.O.C.

The purpose of the present paper is to show the relative importance of thyroid and cortical hormones in the maintenance of normal oxidation and the influence of cortical hormone on the calorigenic action of thyroxine.

METHODS

Experimental. Rats from the colony of this Institute were used. A few days before and during the whole experiment the animals were kept in a heated room (20–24° C.) and allowed free access to their regular stock diet. After the removal of the adrenals (ether anesthesia, dorsal approach) all animals, including the normal or sham operated controls, received Rubin-Krick's salt solution (Rubin & Krick, 1933) as drinking water. Thyroidectomy was also performed under ether anaesthesia.

The oxygen consumption was determined in closed circuit systems (Regnault and Reiset's principle). Each animal was placed in a separate device at 28° C. CO_2 was absorbed by 4% NaOH solution. Before the measurements of O_2 consumption were started, the rats were allowed to adapt themselves for at least 15 min. The oxygen consumption was read in 15–30 min. periods on a gasometer. The surface area of the animals was calculated by means of the formula of Benedict (cited by Du Bois, 1936), and the oxygen consumption was expressed in l./sq.m./hr. The determinations were made daily. When the number of animals was too large, determinations were made every other day. Generally the first measurements showed high values of oxygen consumption, but these values were stabilized after the animals became accustomed to handling. Therefore the first 3–4 determinations were discarded in the computation of the 'normal metabolic level'. (See later.)

The measurements were made without a preliminary fasting period, because adrenalectomized animals generally do not tolerate daily fasts of several hours. Moreover, in previous experiments we had found that in normal rats, the oxygen consumption is not altered by a 24 hr. fast. Similar results have been reported by Carr & Beck (1937), who observed that there was not a significant change in caloric output of normal rats after a fasting period of 48 hr. or after restricting food intake during 10 days.

Computation and representation of the results. Each experiment was divided into several periods. *A* corresponded to the period before adrenalectomy. The mean value of the determinations of metabolism in this period for each group of animals was calculated. This value was called '*normal metabolic level*' (N.M.L.), and was used as zero line in the graph. The percentage variations of the oxygen consumption of the corresponding animal group in the following experimental periods were referred to the respective N.M.L. Period *B* followed adrenalectomy. *C* was a subsequent period during which either cortical extract or thyroxine was administered, and *D* was a final period in which the drugs were withheld. The details of the experiments are given later, with the description of the results. The number of animals surviving on each day is indicated by the figures which accompany the symbols in the graphs.

Drugs used. Thyroxine (Schering) 5 μg./g. of body weight were subcutaneously injected daily. The same amount was given as standard dosage throughout the experiments.

Cortalex (Upjohn) was given with the drinking solution and Eschatin (Parke Davis & Co.) was injected subcutaneously. Doses of cortical hormone are indicated separately for each experiment.

RESULTS

Exp. 1. *Mortality and oxygen consumption of rats after adrenalectomy.* The oxygen consumption of 20 normal male rats weighing between 170 and 250 g. was measured for 8 days (Period *A*), giving a value for the N.M.L. of 9·78 ± 0·625 l./sq.m./hr. (standard deviation). After adrenalectomy (Period *B*) the oxygen consumption was measured, intermittently, for 50 days (Fig. 1).

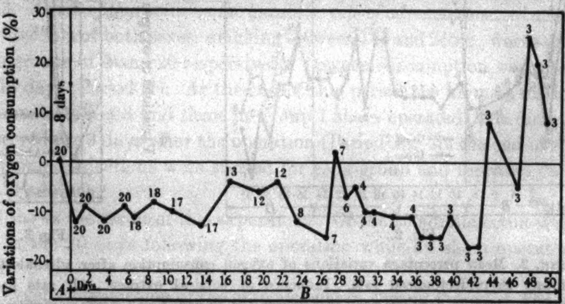

Fig. 1. Exp. 1. Mean percentage variations of oxygen consumption in rats. Period *A*, observation time before adrenalectomy. Period *B*, time after adrenalectomy. The figures accompanying the symbols represent the number of surviving rats.

Between the 22nd and 24th day only half of the rats were alive. Only 3 rats survived after the 34th day, to the end of the observation period. The oxygen consumption of the animals decreased after adrenalectomy below the N.M.L., the maximum decline being 17%. The average decrease of calorigenesis to the 43rd day was 10%. From the 43rd day onwards an increase in the respiratory metabolism of the 3 surviving rats was observed.

Exp. 2. *Influence of adrenalectomy on the oxygen consumption of previously thyroidectomized adult rats and the calorigenic effect of cortical extract.* A preliminary experiment was performed on 17 male rats weighing between 160 and 230 g. which had been thyroidectomized 12 days before starting the measurements of oxygen consumption. They were divided into two groups of 9 and 8 rats respectively. During 6 days following the adrenalectomy there was an average decrease in oxygen consumption of 20% in both groups. At the end of the 6th day treatment with cortical extract (Cortalex, Upjohn) was started, which was added to the drinking solution in a concentration equivalent to 100 mg. fresh organ per c.c. During the period of administration, the mean metabolic rate of the treated animals was 20% higher than that of the untreated, which showed an average decrease of oxygen consumption of 25% after adrenalectomy.

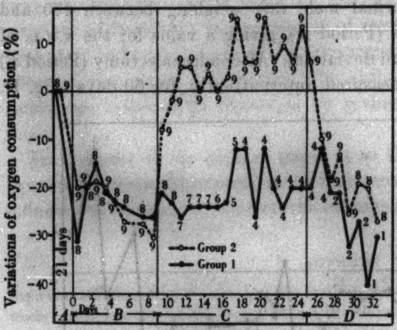

Fig. 2.

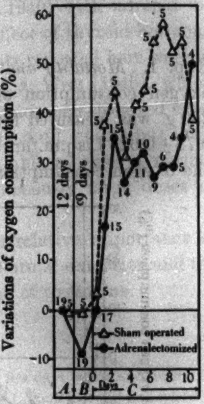

Fig. 3.

Fig. 2. Exp. 2. Mean percentage variations of oxygen consumption after adrenalectomy in previously thyroidectomized rats. Influence of cortical extract. All rats had been thyroidectomized 18 days previous to beginning of Period *A* and adrenalectomized at the end of this period. Group 1, untreated rats. Group 2, rats injected during Period *C* with cortical extract (Eschatin, Parke Davis and Co.). In Period *D* the treatment was discontinued. Figures accompanying the symbols: number of surviving rats.

Fig. 3. Exp. 3. Mean percentage variations of oxygen consumption of normal and adrenalectomized adult rats treated with thyroxine. Period *B*, time after adrenalectomy. During Period *C*, all rats were treated with thyroxine. Figures accompanying the symbols: number of surviving rats.

The results of the second of these experiments are summarized in Fig. 2. Two groups of male rats of 9 and 8 respectively, weighing between 180 and 275 g., were thyroidectomized, and 18 days after this operation the measurements of their oxygen consumption were started, and continued for 21 days (Period *A*). The rats were then adrenalectomized and their metabolism observed during the following 9 days (Period *B*). At the end of this period, Group 2 was treated with Eschatin (3·75 dog units per 100 g. of body weight, diluted with isotonic saline to 1 c.c. and injected subcutaneously). The injections were repeated daily during Period *C*. At the end of the 25th day, the treatment was discontinued. The metabolism of the rats was observed during 8 additional days (Period *D*).

During Period *C* half of the control rats died, only one reaching the end of the experiment, while of the 9 Eschatin treated animals, 8 survived till the end of the observation period (33 days).

The O_2 consumption during Period *A* was, for Group 1, 8·32 ± 0·523 l./sq.m./hr. and for Group 2, 8·78 ± 0·623, but it dropped considerably after adrenalectomy (an average decline of 20% for Group 1 and of 22% for Group 2).

After the first injection of cortical extract, the oxygen consumption in Group 2 was increased and on the third day of treatment reached somewhat higher values than in Period *A*. As soon as the hormone injections were discontinued, the metabolism of this group decreased again, reaching on the 8th day of Period *D* an average decline of 25%.

Exp. 3. *Action of thyroxine on the metabolic rate of adrenalectomized adult rats.* Twenty-five rats of both sexes, weighing between 155 and 245 g., were separated into two groups of 5 and 20 respectively. Oxygen consumption was measured during 12 days (Period *A*). At the end of this period the animals in Group 2 were adrenalectomized and those in Group 1 sham operated. The metabolism was observed for 9 days after the operation (Period *B*). At the end of the 9th day, thyroxine injections were started for each group and repeated daily for 11 days (Period *C*).

Fig. 3 shows the result of this experiment. Of the 20 adrenalectomized rats, 16 died in the 20 days following the operation while all sham operated rats survived.

The oxygen consumption of the animals, which, during Period *A*, had shown values of 9·56 ± 0·834 l./sq.m./hr. for Group 1 and 10·54 ± 0·729 for Group 2, did not vary in the control group after the sham operation, but showed an average decline of 9% in the adrenalectomized animals. During the thyroxine period (*C*) the oxygen consumption of the adrenalectomized rats was somewhat lower than that of the controls, but on the 11th day of Period *C*, the surviving animals showed a metabolic rate 50% above that of Period *A*.

Exp. 4. *Effect of cortical extract on the action of thyroxine in adrenalectomized rats and the influence of adrenalectomy on metabolic rate of animals treated with thyroxine.* In a preliminary experiment 20 male rats, weighing between 120 and 200 g., were separated into four groups of five animals each. The animals in Groups 1 and 2 were adrenalectomized before the administration of thyroxine. Those in Group 2 received treatment with Eschatin during the thyroxine period. Those in Group 3 were adrenalectomized at the height of the thyroxine action, and the remaining rats (Group 4) were sham operated and treated with thyroxine.

The oxygen uptake of rats in Group 1 showed only an unimportant increase after thyroxine administration, but the adrenalectomized Eschatin-treated rats showed a marked increase of oxygen consumption. In Groups 3 and 4 there was a sharp rise of the metabolic rate, but after the 7th day, on which the animals of Group 3 were adrenalectomized, there was a considerable decline of oxygen consumption in these rats. All sham-operated rats increased their metabolism progressively under the action of thyroxine.

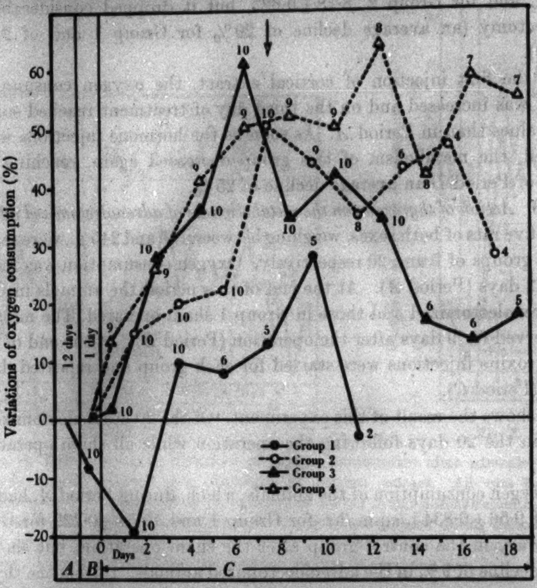

Fig. 4. Exp. 4. Mean percentage variations of oxygen consumption of normal and adrenalectomized rats during treatment with thyroxine. Influence of cortical extract on calorigenic action of thyroxine. All animals were treated during Period *C* with thyroxine. Group 1, 10 rats adrenalectomized at the end of Period *A*. Group 2, 10 rats adrenalectomized at the end of Period *A*, but treated with Eschatin. Group 3, 10 rats adrenalectomized on the 8th day after beginning of thyroxine administration (at the arrow). Group 4, 9 sham-operated rats. Figures accompanying symbols: number of surviving rats.

The experiment represented in Fig. 4 was designed in the same manner as the preliminary experiment, except that a larger number of rats was used. Thirty-nine rats, each weighing between 100 and 235 g., were separated into four groups, Groups 1, 2 and 3 each containing 10 rats and Group 4 containing 9 rats. The N.M.L., measured during 12 days, showed in this period (*A*) an average of 10·42 ± 0·832 l./sq.m./hr. for Group 1, 9·78 ± 0·623 l./sq.m./hr. for Group 2, 9·79 ± 0·835 l./sq.m./hr. for Group 3 and 9·38 ± 0·521 l./sq.m./hr. for

Group 4. At the end of the 12th day, the rats of Groups 1 and 2 were adrenalectomized. On the day following the operation the thyroxine treatment was begun in all groups of rats. During this period the oxygen consumption of each rat was measured every other day.

Rats in Groups 1 and 2 were adrenalectomized before the thyroxine period, and those in Group 1 received daily subcutaneous injections of 1 c.c. of isotonic NaCl, while those in Group 2 were treated with Eschatin during the thyroxine administration (20 dog units per rat, made up to 1 c.c. with isotonic NaCl, were given daily). Rats in Group 3 were adrenalectomized on the 8th day following thyroxine administration, while those in Group 4 received thyroxine but did not undergo operation.

Thyroxine caused a high mortality in the animals adrenalectomized in Period *B*. On the 14th day after the beginning of thyroxine injections, all rats of Group 1 were dead. The rats of Group 2, adrenalectomized simultaneously with Group 1 but treated with cortical extract, were more resistant to the lethal effect of thyroxine (4 of the 10 rats survived up to the 17th day). The 10 rats of Group 3, adrenalectomized during the height of thyroxine action, survived the operation for 4 days. Five of these animals reached the end of the experiment. Of the 9 rats of the control Group 4, one died on the 13th day and another on the 17th day of Period *C*.

Throughout the thyroxine period, the oxygen consumption in Group 1 was lower than that in all other groups. The metabolic rate of the Eschatin-treated animals in Group 2 increased with thyroxine. The rats of Group 3 first showed a normal metabolic reaction to thyroxine but after adrenalectomy, and in spite of continued thyroxine administration, there was a continuous decrease of oxygen consumption. The non-operated controls of Group 4 showed an increase of their metabolism by more than 50% at the end of the experiment.

Exp. 5. *The effect of thyroxine on the oxygen usage of infantile adrenalectomized rats.* Figs. 5 and 6 represent two experiments. The first (Fig. 5) was carried out on 23 male rats each weighing between 70 and 100 g. Four rats served as controls, while 19 were adrenalectomized after a 6-day period of measurements of N.M.L. The average oxygen consumption in Period *A* was 10·82 ± 1·042 l./sq.m./hr. for the controls and 10·22 ± 0·728 l./sq.m./hr. for the remaining rats. At the end of the 5th day following the adrenalectomy the treatment with thyroxine was begun on all animals. During the thyroxine period (*C*), 18 of the 19 adrenalectomized rats died, while the controls survived the 6-day treatment. The oxygen consumption of the adrenalectomized rats was considerably lower than that of the controls. The 4 rats which survived the 5th day of the thyroxine period reached an 18% increase of metabolic rate, while the controls raised their oxygen consumption by 49%.

The second of these experiments (Fig. 6) was designed like the preceding experiment, except that the number of rats, especially of the controls, was larger and the observation time longer. Seventeen of the 22 adrenalectomized rats died and 3 of the 10 non-operated controls. The average N.M.L. of the control group was 11·22 ± 0·935 l./sq.m./hr. and that of the other group 11·62 ± 1·018 l./sq.m./hr. The oxygen consumption of the operated rats was low after thyroxine treatment, with the exception of the metabolic reaction observed on the third day of Period *C*, when the 11 surviving rats showed an average increase of 27 % in their oxygen consumption.

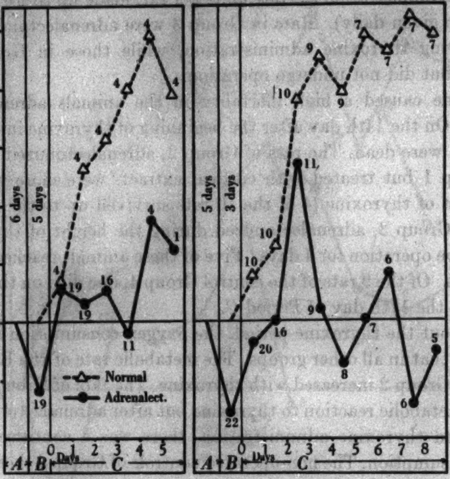

Fig. 5. Fig. 6.

Figs. 5 and 6. Exp. 5. Mean percentage variations of oxygen consumption of young normal and adrenalectomized rats during thyroxine administration. During Period *C*, thyroxine was injected. Figures accompanying symbols: number of surviving rats.

Exp. 6. *The effect of thyroxine in adult thyroidectomized-adrenalectomized rats.* Twenty-eight male rats, each weighing between 185 and 240 g., were thyroidectomized, and 25 days after this operation the measurements of their oxygen consumption were started. They were separated into two groups (Group 1 of 20 rats and Group 2 of 8). The metabolic rate was determined for 8 days (Period *A*) after which all animals were adrenalectomized (Period *B*). At the end of the 3rd day thyroxine injections were begun (Period *C*). Rats in Group 2 were treated with cortical hormone in addition to thyroxine. During the first 3 days of Period *C*, 5 dog units of Eschatin per 100 g. of body weight, diluted to 1 c.c. with isotonic NaCl was injected twice daily. After the third day the Eschatin dose was reduced to one-half. The controls received 1 c.c. of isotonic NaCl solution twice daily (Fig. 7).

Of the 20 rats in Group 1, 19 died between the 2nd and 8th day after the beginning of Period *C*. The rats in Group 2, injected with Eschatin, lived for 4 days with thyroxine treatment, but after lowering the hormone supply, all 8 rats died within 2 days (Fig. 7).

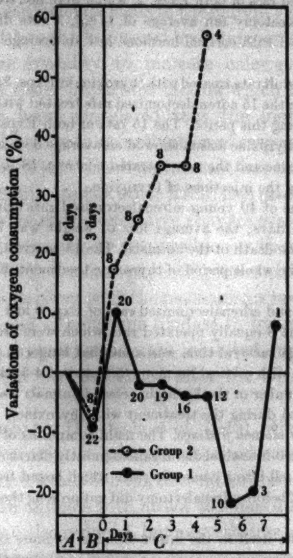

Fig. 7. Exp. 6. Mean percentage variations of oxygen consumption of thyroidectomized-adrenalectomized adult rats treated with thyroxine. All animals had been thyroidectomized 25 days previously to Period *A*, adrenalectomized at the end of Period *A* and injected with thyroxine during Period *C*. Group 1, 20 rats injected with isotonic NaCl solution. Group 2, 8 animals treated with Eschatin. Figures accompanying symbols: number of surviving rats.

The metabolic rate of the animals, which showed an average value of 9·18 ± 0·623 l./sq.m./hr. in Group 1 and 9·76 ± 0·936 l./sq.m./hr. in Group 2 during Period *A*, decreased after the adrenalectomy (Period *B*). The oxygen consumption of the rats which did not receive cortical hormone therapy showed a slight increase after the first thyroxine injection, but decreased during the following days. On the other hand, the Eschatin-treated rats showed a considerable calorigenic response to thyroxine, increasing their oxygen consumption by more than 50%.

General remarks concerning all experiments

Weight changes. Of the 20 adrenalectomized rats of Exp. 1, 6 showed either no decrease in weight or a slight increase, 11 rats lost weight (an average of 12%), and the 3 rats that survived the operation showed, on the 50th day after the operation, increases of weight of 38, 44 and 50% respectively.

Of the 17 thyroidectomized rats of both Exps. 2, which were not treated with cortical hormone, 13 lost weight after adrenalectomy (an average of 11%), 4 rats did not change their weight. Fifteen of the 17 rats treated with cortical hormone lost an average of 7% and 2 animals maintained their weight.

The 35 adrenalectomized adult rats treated with thyroxine in Exps. 3 and 4 lost an average of 13% during the thyroxine period; the 15 adrenalectomized rats treated with cortical hormone in Exp. 4 lost 9% of their weight during this period. The 15 rats of both Exps. 4 which had been adrenalectomized at the height of thyroxine action showed an average decrease in weight of 10% during the administration of thyroxine and the non-operated controls, 18 in all from Exps. 3 and 4, did not alter their weight during the injections of thyroxine.

The average survival time of 40 young adrenalectomized rats (Exp. 5) after the beginning of thyroxine treatment was 4 days; the average loss of weight was 7% (from the beginning of thyroxine injections up to the death of the animals). The 14 controls, which survived longer, lost 3% of their weight during the whole period of thyroxine treatment, but during the first 4 days the average decrease was 6%.

The 20 thyroidectomized and adrenalectomized rats of Exp. 6 lost 13% of their weight during the thyroxine treatment, the 8 equally operated rats which were treated with Eschatin lost an average of 3%. (The average survival time was somewhat longer in the non-treated group than that of the animals injected with cortical hormone (5·2 against 4·5 days), which perhaps was due to the fact that, first, the number of the Eschatin-treated animals was smaller, and secondly, the dose of Eschatin was reduced during the treatment with thyroxine.)

Systemic reactions and post-mortem findings. The main symptoms of adrenal insufficiency in our rats were adynamia and gastro-intestinal disturbances (mostly diarrhoea). Death usually occurred very suddenly; the animals fell into a comatose state which lasted from half an hour to an hour.

Thyroidectomy performed before adrenalectomy did not prolong the survival time of the animals, as can be seen from Exp. 2.

Hyperthyroidism manifests itself in the intact animals by more or less pronounced asthenia, diarrhoea, tachycardia and peripheral vasodilatation (red ears), but these symptoms are not very marked with the dose of thyroxine used in the above-described experiments. In the adrenalectomized rats treated with thyroxine these systemic reactions appear earlier and are considerably more severe. The lower the caloric output of these rats, the more severe are the thyrotoxic symptoms. Convulsions very frequently precede death.

Post-mortem examination showed extremely large thymus; complete loss of fat depots, especially marked in the perirenal adipose tissue; the interscapular fatty organ appeared dark red. The heart showed almost immediate rigor mortis.

DISCUSSION

There are several possible mechanisms to be considered, which could explain the decreased effect of thyroid hormone on oxygen usage in adrenal insufficiency. The simplest explanation of the phenomenon would be the assumption that, due to the lowered vitality of the animals, an increase in metabolic rate could not be obtained. On the other hand, rats with intact adrenals, when treated with large doses of thyroxine for long periods, enter into a state of thyrotoxic crisis, during which time they show a loss of weight, considerable myasthenia and circulatory disturbances; nevertheless, their metabolism is raised to more than twice its normal level (personal observations). The result of Exp. 5 also shows that on the day preceding death the Eschatin-treated rats had considerably increased their caloric output.

The hypothermia which is regularly observed in adrenalectomized rats suggests that adrenal insufficiency leads to the inability of the animal to increase its heat production. Although the lowered body temperature could undoubtedly be responsible for the inability to increase calorigenesis after thyroxine, apparently there exist other important factors which have to be discussed.

The possibility has been considered that the increase of oxidations in hyperthyroidism could be due to an activation of respiratory enzymes (Tipton, Leath, Tipton & Nixon, 1946). Such an increase has been observed (Gerard & Cohen, 1937; Klein, 1939). On the other hand, it is also known that in adrenal insufficiency disturbances of the oxidative enzyme systems appear, and it has been suggested that these may be responsible for the decrease in basal heat production (Tipton, 1941). A recent paper (Tipton *et al.* 1946) provides evidence that cortical hormone interferes with the mechanisms responsible for the activation of succinoxidase and cytochrome oxidase in hyperthyroidism. Adrenalectomy performed on hyperthyroid rats inhibited further increase of these enzymes, but activation was evidenced after the administration of cortical hormone.

Verzar and his associates (Doetsch *et al.* 1945), who observed a very low calorigenic effect of thyroxine in adrenalectomized cats, believe that 'if there are no oxidizable reserves available, the thyroid hormone will have no influence on O_2 consumption'. In animals with intact adrenals during thyrotoxicosis the 'oxidizable reserves' are also reduced, but nevertheless metabolism is extremely high. Considering the increased nitrogen output, it may reasonably be deduced that the hyperthyroid organism draws on its own tissue proteins and produces readily oxidizable nutrients by increasing gluconeogenesis (Wells & Kendall, 1940). Possibly the high caloric output in hyperthyroidism may be in part ascribed to the increased protein utilization ('S.D.A.' of cell proteins). In several instances the belief has been emphasized that there is no reason to disregard a possible effect of catabolized body proteins upon energy metabolism. (References summarized by Forbes, Swift, Elliot & James, 1946.)

In adrenal insufficiency, on the other hand, the utilization of protein breakdown products seems to be disturbed. v. Arvay (1929) observed a considerable decrease of S.D.A. of nutrients in rats showing severe symptoms of adrenal insufficiency. The results of Brownell & Hartman (1941) also suggest that after adrenalectomy the calorigenic processes induced by feeding are disturbed.

The rate of deamination is lowered in kidney slices from adrenalectomized cats (Jiménez Díaz, 1936) and rats (Russell & Wilhelmi, 1941). The decreased ability of the tissues to perform their normal metabolic activity seems to become especially evident when adrenalectomized animals are required to expend energy at accelerated rates, i.e. when they are subjected to stress. After adrenalectomy, fasted phlorizin-diabetic rats showed less gluconeogenesis than animals treated with hormones of the corticosterone type (Long, Katzin & Fry, 1940; Wells, 1940; Evans, 1936). Especially interesting are the experiments performed by Wells & Kendall (1940) on adrenalectomized rats which previously had been submitted to thyroidectomy; the combination of both operations resulted in a much lower rate of gluconeogenesis than did either operation performed separately.

Correlating these observations with ours one may conclude that apparently there is a quantitative relation between the rate of gluconeogenesis and the energy output of the animals. Possibly the low calorigenesis in thyroxine-treated adrenalectomized rats is due to their inability to synthesize glucose from proteins. In other words, in the absence of cortical hormone, thyroxine would not adequately stimulate the energy-yielding processes in the body.

The above-described results suggest that there is a direct relation between the intensity of the metabolic action of thyroxine and the amount of available cortical hormone. In Exps. 3 and 4 the calorigenic effect of thyroxine was still clearly manifest although reduced in the adrenalectomized animals, whereas in Exps. 5 and 6 it was extraordinarily low. A reasonable explanation for these differences is that Exp. 3 and 4 were performed on adult rats, which were able to compensate for adrenal insufficiency by activation of accessory cortical tissue. It is also possible that the marked decline of O_2 consumption in Exp. 2 and the decreased calorigenic effect of thyroxine which characterized Exp. 6 are caused by the lack of thyroid secretion which leads to an atrophy of the adrenals and of the accessory cortical tissue. It may be assumed that in previously thyroidectomized rats there remains after the extirpation of the principal adrenal organ an accessory tissue with low secretory activity. In support of this interpretation, observations could be cited which indicate degenerative changes of the adrenal cortex after thyroidectomy (Gley, 1913; McCarrison, 1922).

Exps. 2 and 3, performed on thyroidectomized-adrenalectomized rats, suggest that in these animals the adrenal cortex is more essential than the thyroid gland for the maintenance of the metabolic level. In Exp. 6, thyroxine did not significantly raise the caloric output, while in Exp. 2, in absence of thyroid secretion, cortical extract raised the low metabolic level. Webster *et al.* (1932) also reported an increase of caloric output of thyroidectomized and adrenalectomized cats after treatment with cortical extract. They even observed that large doses of cortical hormone increased the metabolism of myxedematous animals with intact adrenals. As expressed by the authors, this may represent 'an attempt on the part of the adrenal cortical hormone to compensate for the deficiency of thyroxine'. On the other hand, it does not seem unreasonable to assume that the decrease in metabolic rate after thyroidectomy is the result of decreased activity of the adrenal cortex, which may be compensated by giving cortical hormone.

It is a well-established fact that administration of thyroid hormone leads to hypertrophy of the adrenal cortex (Cohen, 1935; Gerlei, 1937; Herring, 1917; Hoen & Oehme, 1938; Hoskins, 1910; Miller & Riddle, 1939; Schmidt & Schmidt, 1938; Squier & Grabfield, 1922; Uotila, 1940), and there is evidence that this morphological change actually expresses increased function (Lowenstein & Zwemer, 1942). Possibly the normal resistance to an excess of thyroxine is dependent upon an increase of cortical hormone output. The idea expressed by Tepperman, Engel & Long (1943) is suggestive that the stimulus for adrenal hyperfunction after thyroid administration is not the hormone itself but the products of increased protein catabolism produced by it.

SUMMARY

1. Adrenalectomy performed on adult rats decreases the oxygen consumption by an average of 10%.

2. Adrenalectomy performed on previously thyroidectomized adult rats is followed by an average decline of metabolic rate of more than 20%. Administration of cortical extract raises the metabolic rate of thyroidectomized-adrenalectomized rats.

3. The administration of thyroxine to adrenalectomized rats increases the mortality rate.

4. The effect of thyroxine on metabolic rate is decreased in adrenalectomized rats. The inability to react to thyroxine with an increase of oxygen consumption is especially pronounced in young animals and in thyroidectomized adult rats.

5. Adrenalectomy performed during the height of thyroxine action leads to a decrease of oxygen consumption, in spite of continuous thyroxine administration.

6. Administration of cortical extract to adrenalectomized rats raises to normal their metabolic response to thyroxine.

7. The mechanisms involved in the action of cortical hormone and of thyroxine are discussed.

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THE INFLUENCE OF OVARIAN HORMONES ON THE TRANSMISSION OF THE ACTIVITY OF THE SYMPATHETIC NERVES OF THE CAT

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Secker (1938) reported that as a result of adrenalectomy in cats the nictitating membrane (n.m.) failed to retract on stimulation of the cervical sympathetic nerve but could be restored to normal activity by the administration of adrenocortical extracts. This failure to respond was obtained invariably in male cats, but in some female cats it occurred only when the ovaries had been removed as well. This fact seemed to point to some functional relationship between the suprarenals and the ovaries. There are many observations which suggest that the corpus luteum can act as an effective substitute for the suprarenal cortex, such as the prolonged survival time of adrenalectomized animals during pregnancy and pseudo-pregnancy or on injections of sufficient amounts of progesterone and the finding that adrenalectomized pregnant animals can live till parturition free from any signs of adrenal insufficiency.

The work reported here is an attempt to discover whether progesterone, as well as cortical hormone, is capable of restoring the response of the n.m. to sympathetic nerve stimulation in adrenalectomized animals and to obtain an explanation for the inconstant results obtained on female cats.

METHOD

The experimental procedure has been described elsewhere (Secker, 1938). Bilateral ovariectomy was carried out either at the time of the adrenalectomy, or at a later stage of the experiment when it proved impossible to obtain a reduction or failure of the response of the n.m. on stimulation of the cervical sympathetic even after it had been stimulated for a prolonged period. The female cats were grouped according to the stage of the sexual cycle shown by the ovaries: Group P (an- or pro-oestrus) were cats whose ovaries contained follicles in an early stage of development or with degenerating corpora lutea. Group O (oestrus) were cats with very large engorged follicles, i.e. in the active stage of oestrus. Group R were pregnant or lactating animals.

The following preparations of female sex hormones were used for injection: 1 ml. Progestin 'B.D.H.' (1 mg. progesterone/ml.; 1 i.u.); 1 ml. Lutocyclin 'Ciba' (2 mg. anhydro-hydroxyprogesterone in 1 ml. sesame oil); 2 ml. Theelin, Parke Davis and Co (200 units/ml.); and 1 ml. Ovocyclin 'Ciba' (oestradiol di-propionate, 1 mg./ml. contains 10,000 di-propionate units). The hormones in oil were given either intramuscularly or intravenously after shaking with saline.

RESULTS

As shown previously by Secker, in male suprarenalectomized cats, stimulation of the cervical sympathetic leads to a condition in which the contraction of the n.m. is no longer maintained. This observation could be confirmed. The time of stimulation necessary to produce the effect in the present experiments varied from 1·5 to 14·5 min. (mean 7 min.) in different cats. After such a period of stimulation renewed stimulation produced a small effect only. In three out of fifteen adrenalectomized cats this reduction in the response of the n.m. could not be obtained; two of these cats were long-standing castrates.

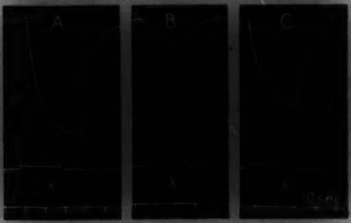

Fig. 1. Contractions of the right n.m. in female cat under dial anaesthesia. At X, 20 sec. stimulation of right cervical sympathetic. A, 30 min. after adrenalectomy. Between A and B, stimulation of cervical sympathetic for total period of 10·5 min. C, 7 min. after injection of Eucortone. Time in 10 sec.

Fig. 1 is the record of an experiment on a female adrenalectomized cat in which the result is similar to that usually obtained in the male. Stimulation of the cervical sympathetic for a total period of 10 min. (between A and B) produces a condition in which subsequent stimulation of the cervical sympathetic gives a greatly reduced contraction of the n.m. As in the male animal, recovery of the response to nerve stimulation can be obtained by an injection of cortical extract. For instance, in the experiment (Fig. 1), 1 ml. of Eucortone (Allen and Hanbury Ltd.) was injected intravenously between B and C, 5 min. before the cervical sympathetic was restimulated in C. 1 ml. of Eucortone is equivalent to 75 mg. of tissue of the suprarenal cortex. Similar results were obtained on injection of 2 ml. Eschatin (Parke Davis and Co.), equivalent to 80 mg. tissue. Histological examination of the ovaries of this cat showed many follicles in the course of enlargement, but corpora lutea were absent.

Results similar to those illustrated in Fig. 1 were obtained in all adrenalectomized cats which belonged to group P (fifteen cats) and in those cats in which ovariectomy was performed at the time of adrenalectomy (nine cats). These cats were either pregnant, lactating or belonged to group O.

In two cats, one belonging to group P and one to group R, adrenalectomy with or without ovariectomy respectively did not seem to produce the reduction in the response to stimulation of the cervical sympathetic. But when the site of the adrenal ligature was inspected a small pedicle of cortex was found outside the ligature. On removal of the pedicle, which, on histological examination, proved to be cortical tissue the failure of the n.m. to respond to nerve stimulation was at once evident.

Fig. 2. Contractions of the right n.m. in pregnant adrenalectomized cat under dial anaesthesia. At *X*, 20 sec. stimulation of right cervical sympathetic. Between A and B cervical sympathetic stimulation for a total period of 17 min. Between B and C removal of uterus and ovaries and renewed stimulation of cervical sympathetic for a total period of 16 min. Between C and D injection of 1 ml. Eucortone. Time in 10 sec.

In one lactating cat, in two pregnant cats and in seven belonging to group O adrenalectomy was performed without simultaneous removal of the ovaries. In none of these animals was it possible to obtain a reduction, or more than an insignificant reduction, of the response of the n.m. on stimulation of the cervical sympathetic, but the phenomenon could easily be obtained on subsequent removal of the ovaries. This is illustrated for a pregnant cat, the ovaries of which showed several large corpora lutea, by the experiment of Fig. 2. Stimulation of the cervical sympathetic for a total period of 17 min., between A and B, did not bring about the condition of failure of the response of the n.m. on subsequent stimulation, although both suprarenals had been removed. In the interval between B and C the uterus and the ovaries were removed whilst the cervical sympathetic was stimulated for a further period of 16 min. Later, at C, the response to stimulation of the cervical sympathetic was greatly reduced. Subsequent recovery was obtained after the intravenous injection of 1 ml. Eucortone (between C and D).

The time of stimulation of the cervical sympathetic necessary to produce the reduced response of the n.m. corresponded to that found in male adrenalectomized cats. It varied between 0·5 and 26·5 min. (mean 8 min.). There was no difference between those female cats belonging to group P, in which adrenalectomy only was performed and those in which the ovaries were removed as well.

Fig. 3. Contractions of the right n.m. in adrenalectomized female cat under dial anaesthesia. At X, 30 sec. stimulation of right cervical sympathetic. Between A and B stimulation of cervical sympathetic for a total period of 24 min. C, 5 min. after injection of 1 ml. Progestin. Time in 10 sec.

Fig. 4. Contractions of the right n.m. in male cat under dial anaesthesia. At X, 20 sec. stimulation of cervical sympathetic. A, 20 min. after adrenalectomy. Between A and B stimulation of cervical sympathetic for a total period of 16 min. Between B and C injection of 1 mg. Progestin. Time in 10 sec.

Corpus luteum hormone preparation. Progestin, as well as Lutocyclin, were effective in restoring the response of the n.m. Observations were made on six female adrenalectomized cats, and if they belonged to group O or R the ovaries had been removed as well. Fig. 3 illustrates the effect of Progestin in a female cat belonging to group P. In the interval between A and B the cervical sympathetic was stimulated for periods amounting to 24 min. This caused the reduction in the response of the n.m. seen at B, but practically full recovery was restored at C, 5 min. after the injection of 1 ml. Progestin.

Corpus luteum hormone preparations exerted the same effect also on adrenalectomized male cats. Seven cats were examined for this purpose and Fig. 4 is a typical record from one of the experiments. In the interval between A and B the cervical sympathetic nerve had been stimulated for a total period of 16 min.; between B and C, 1 ml. Progestin was injected.

Fig. 5. Contractions of the right n.m. of lactating adrenalectomized cat. At X, 30 sec. stimulation of cervical sympathetic. Between A and B stimulation of cervical sympathetic in a total period of 17 min. Between B and C injection of 2 ml. Theelin, stimulation at C, 7 min. after injection. Time in 10 sec.

Follicle hormone preparations. Both Theelin and Ovocyclin were able to restore the response of the n.m. in adrenalectomized cats, but the effect was weaker than that of progesterone or of cortical extracts. Observations were made on eight female and on four male cats. Fig. 5 shows the effect of 2 ml. Theelin in restoring the response of the n.m. to sympathetic stimulation in a lactating cat (group R) deprived of its ovaries and suprarenals at the beginning of the experiment. The reduction in the response of the n.m. at B was brought about by stimulation of the cervical sympathetic for a total period of 17 min. between A and B. Record C was obtained 7 min. after the injection of 2 ml. Theelin.

DISCUSSION

The original observations of Secker were confirmed. In adrenalectomized male cats stimulation of the cervical sympathetic for a few minutes reduced the response of the nictitating membrane to subsequent nerve stimulation. In female cats this effect could only be obtained in those cats the ovaries of which were in a condition of an- or pro-oestrus. In oestrus, during pregnancy and lactation, the ovaries had to be removed as well. These results suggested that the female sex hormones, particularly the corpus luteum hormone, are able to replace the cortical hormone which appears necessary for the normal functioning of the sympathetic nervous system. This suggestion was strongly supported by the finding that it was possible in female as well as in male cats to restore the activity of the sympathetic nerve on the n.m. by injections of corpus luteum hormone preparations. Preparations of follicular hormone were also effective but not to the same extent.

These results therefore strongly support Secker's original conception that cortical hormone is necessary for the normal functioning of the sympathetic system and explains the variability of the results obtained in adrenalectomized female cats with intact ovaries.

Two groups of workers had failed to substantiate these ideas. Armstrong, Cleghorn, Fowler & McVicar (1939), in a series of experiments on adrenalectomized cats in the terminal stage of insufficiency or maintained in health for some weeks by cortical extract, could find no impairment of the retraction of the n.m. on stimulation of the cervical sympathetic. If one discards the results on female cats and examines the protocols of the two male cats reported (Exp. 5, cat 22; and Exp. 8, cat 125) one finds the conditions of the experiment different from those of Secker. Both cats had been maintained for some time on cortical extract which was withdrawn 9 days and 14 days respectively before stimulation of the cervical sympathetic. The cats were then prostrate, or nearly so. Secker's experiments were all acute, and no cortical extract was given till the n.m. had failed to respond. Further, in cat 125 they appear to have stimulated the sympathetic after the injection of adrenaline (0·04 mg./3·4 kg.). Secker (1938, p. 268) showed clearly that the injection of 0·03 mg./kg. of adrenaline could temporarily restore the contractility of the n.m. to sympathetic stimulation.

Haterius & Maison (1942) repeated Secker's experiments using ten male, four female and two ovariectomized cats, and failed to get any diminution in the retraction of the n.m. in response to stimulation of the cervical sympathetic after acute adrenalectomy, except in the case of two animals, one male and one female, which were so nearly moribund that adrenaline (i.v.) failed to elicit a response. The only differences between their experiments and those of Secker were in their use of urethane as anaesthetic and in that they cut only the ipsilateral vagus trunk while Secker cut both vagi.

It is not possible to explain satisfactorily the difference between Secker's results and those of Haterius & Maison. The possibility, however, cannot be excluded that the difference in the anaesthetic is responsible for this inability to confirm Secker's observations. It is well known that different anaesthetics fundamentally influence the reactivity of autonomic nerve effects.

SUMMARY

1. In male cats, under dial anaesthesia, acute adrenalectomy usually leads to a condition in which the response of the nictitating membrane to sympathetic stimulation becomes quickly reduced and remains reduced on renewed stimulation.

2. In female cats, under dial anaesthesia, adrenalectomy produces this reduced response to sympathetic stimulation only when the ovaries are in a condition of an- or pro-oestrus. In oestrus, and during pregnancy and lactation, the ovaries have to be removed as well.

3. The reduced response of the n.m. to sympathetic nerve stimulation in adrenalectomized male and female cats can be restored by the injection of preparations of cortical hormone. The necessity of removal of the ovaries as well as the adrenals during oestrus and pregnancy, in order to obtain the condition of a deficiency in the response to sympathetic stimulation, suggested that the female sex hormones can replace the cortical hormones in their action on the sympathetic nervous system. This was borne out by the fact that progesterone caused a restoration, similar to that of cortical extract, of the reduced response of the n.m. to sympathetic stimulation in adrenalectomized male and female cats. Follicular hormone had a similar action, but the effect was weaker than that of progesterone or cortical extract.

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THE RELATIONSHIP BETWEEN THE CHANGE IN THE ELECTRORETINOGRAM AND THE SUBJECTIVE DARK-ADAPTATION CURVE

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It is well known that the electroretinogram (E.R.G.) changes appreciably during dark adaptation. Granit (1938) has shown that the nature of the change depends to some extent on whether the eye in question possesses an E- or I-type of retina and he has further demonstrated (Granit, 1933) that, so far as the electroretinogram is concerned, the human eye has an E-retina. In the human electroretinogram dark adaptation reduces or obliterates the small negative *a*-wave and causes a considerable increase in the size of the *b*-wave, while the whole electrical response is much prolonged (Cooper, Creed & Granit, 1933; Karpe, 1945). These changes can be seen in Fig. 1.

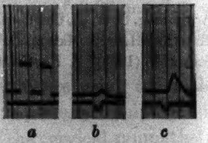

Fig. 1. The effect of the state of adaptation on the *b*-wave of the human electroretinogram. *a*, Calibration to 0·5 mV. *b*, E.R.G. of an eye adapted to 5 m.c. *c*, E.R.G. of an eye adapted to about 0·05 m.c. Stimulus 20 m.c. restricted to a central retinal area subtending 8°.

The increase in sensitivity of an eye which occurs during dark adaptation is usually expressed in terms of the light intensity just visible (the threshold) at any given moment, and when the threshold is plotted against the duration of dark adaptation the well-known dark-adaptation curve shown in Fig. 2 is obtained. This curve is made up of two parts, the first, lasting for about 7 min. in Fig. 2, being generally considered to represent cone adaptation and the second rod adaptation.

Great improvements in the technique of recording the human electroretinogram have now made it possible to follow its changes during dark adaptation with fair accuracy, and the time seems to have come to consider the relationship between these changes and the subjective dark-adaptation curve. Is any relationship, in fact, demonstrable and, if so, what does it mean in terms of the process of dark adaptation?

METHOD

The subjective dark-adaptation curve and the change in size of the *b*-wave were recorded simultaneously for the left eye of one of us (G. K.).

Gullstrand's method, using Carle's apparatus (Carle, 1937), was used to determine the subjective curve; this method measures the light threshold only; no form or brightness discrimination is required. The technique recently described by one of us (Karpe, 1945) was used for recording the electroretinogram.

In order to ensure that the initial condition of light adaptation was as constant as possible from one experiment to another, the experiment was begun by a preliminary period of dark adaptation which was continued until a fairly constant threshold value was obtained. The light adaptation consisted of exposure to 5000 m.c. for 30 sec. followed by 10,000 m.c. for 5 min. The final dark-adaptation curves were then taken.

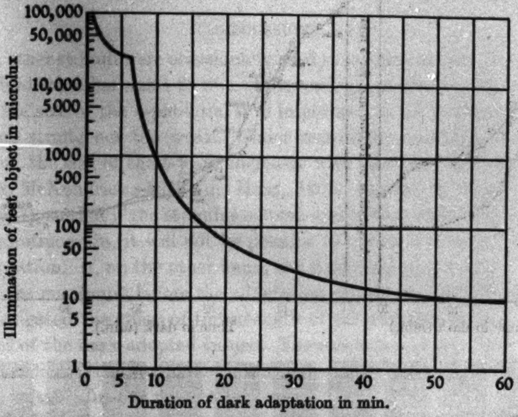

Fig. 2. The subjective dark-adaptation curve. Abscissae: duration of dark adaptation in min. Ordinates: the smallest visible illumination in microlux (1,000,000 microlux = 1 m.c.). (After Kohlrausch (1931). See Wright & Granit (1938).)

After the subject had been prepared for electroretinography with a contact glass on the eye to be investigated, the white screen of Carle's apparatus was placed in front of him with a red fixation light so that the subjective stimulus fell 12° from the fovea on one side and the E.R.G. stimulus 8° on the other. The room lights were then extinguished and the subject indicated as soon as the subjective stimulus became visible. This was then reduced, the time having been noted, and he again indicated as soon as he could see it. At the same time the E.R.G. to a constant stimulus of 1·25 m.c. was recorded, first at intervals of 1 min., later at 2 min. intervals and finally (after 30 min.) at 5 min. intervals.

When dark adaptation seemed to be reasonably complete a triple ground glass screen was placed close to the subject's face and a 500 W. bulb lit behind it, followed after 30 sec. by another 500 W. bulb. One bulb alone gave an illumination of 5000 m.c. and both together 10,000 m.c. evenly distributed over practically the whole field of vision. After 5 min. both lamps were put out, the ground glass removed and the final recording of the two dark-adaptation curves begun. This usually lasted for 40–60 min.

RESULTS

The results are shown in the curves given in Fig. 3, which illustrate the course of one whole experiment comprising two dark adaptations separated by as intense a light adaptation as we could produce.

The plotting of two curves concerned with such different values as the subjective threshold illumination and the height of the *b*-wave of the electroretinogram on one figure is certain to present some difficulty in the selection of the scales to be compared. However, in the present instance, we have one common point in the asymptotic value finally reached by both curves: the maximum *b*-potential of nearly 0·30 mV. and the subjective threshold of about 0·001 m.c. (the high subjective threshold is due to the presence of the contact glass with possibly some corneal oedema; without a contact glass the same eye normally had a threshold of 0·00003 m.c.). There is a good deal of evidence (see

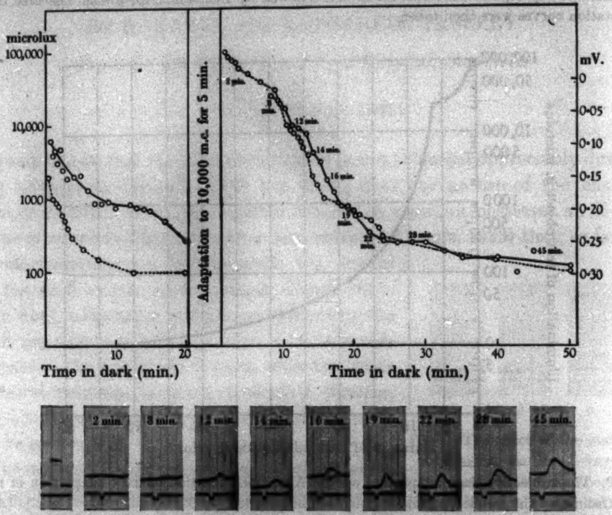

Fig. 3. Comparison of the subjective dark-adaptation curve and the *b*-potential dark-adaptation curve of the same eye before and after intense light adaptation. o- - - - -o subjective curve; o———o *b*-potential curve. Abscissae: duration of dark adaptation. Ordinates: *Left*, Log illumination in microlux; *Right*, *b*-potential in mV. 5 min. light adaptation to 10,000 m.c. between the two dark adaptations. A selection of the E.R.G.'s recorded after the light adaptation preceded by a calibration to 0·5 mV. is given at the bottom of the figure. The times at which these were recorded are given both on the records and on the curve. Stimulus for E.R.G., 1·25 m.c.

Granit, 1947) suggesting that there is a particularly close association between the *b*-potential and rod activity, and this seemed a good reason for placing the 0 value of the *b*-potential curve at the position of the kink in the subjective curve, where rod adaptation is generally thought first to become manifest. When these two points on each curve are placed together, the *b*-wave and subjective dark-adaptation curves following an efficient light adaptation show a surprisingly good empirical fit. When stronger stimuli (20 or 80 m.c.) were used, however, the two curves did not fit so well, for after light adaptation the *b*-wave curve fell more rapidly than the subjective one. With the 80 m.c. stimulus, there was, on the other hand, a fairly good fit between the two curves taken *before* light adaptation (Fig. 4).

DISCUSSION

Although other stimuli were occasionally used to produce an electroretinogram, our standard stimulus was 1·25 m.c. In recording a dark-adaptation curve in terms of the size of the *b*-potential it is important to use a stimulus which is neither too strong nor too weak. Under reasonably constant conditions of adaptation, the size of the *b*-wave increases with increased stimulus intensity (Dewar & McKendrick, 1873; de Haas, 1903; Chaffee & Hampson, 1924; Granit, 1947), so that if the stimulus is weak and lies too near the threshold for the electroretinogram, it will not be possible to obtain a *b*-wave which alters with adaptation. If, on the other hand, the stimulus is too strong, the *b*-wave will reach its maximum before the adaptation process is complete. We, therefore, investigated the effect of the strength of the stimulus on the size of the *b*-potential of the dark-adapted subject. The results are shown in the curve of Fig. 5 and indicate that a stimulus of 1·25 m.c. from the rising part of the curve should be suitable for our present purpose.

It is clear from the curves given in Fig. 3 that the increase in the size of the *b*-potential evoked by a constant stimulus can be used as a measure of the course of dark adaptation in man. With an efficient preceding light adaptation and a suitably selected stimulus, the curve relating the size of the *b*-potential to time in the dark follows at least the latter part of the sensory dark-adaptation curve very closely indeed. Under these conditions this curve does appear to correspond to what is generally known as the rod part of the ordinary subjective dark-adaptation curve.

In this connexion it is of interest to mention some other reasons for supposing that the electroretinogram, taken with this apparatus and this stimulus (1·25 lux), is probably dominated by the reactions of the rods. For instance, the presence of macular degeneration is not associated with an abnormal electroretinogram whereas retinitis pigmentosa, even where good central vision is still present, is characterized by a response which is very much diminished or even absent. Perhaps of greater significance is the fact that we

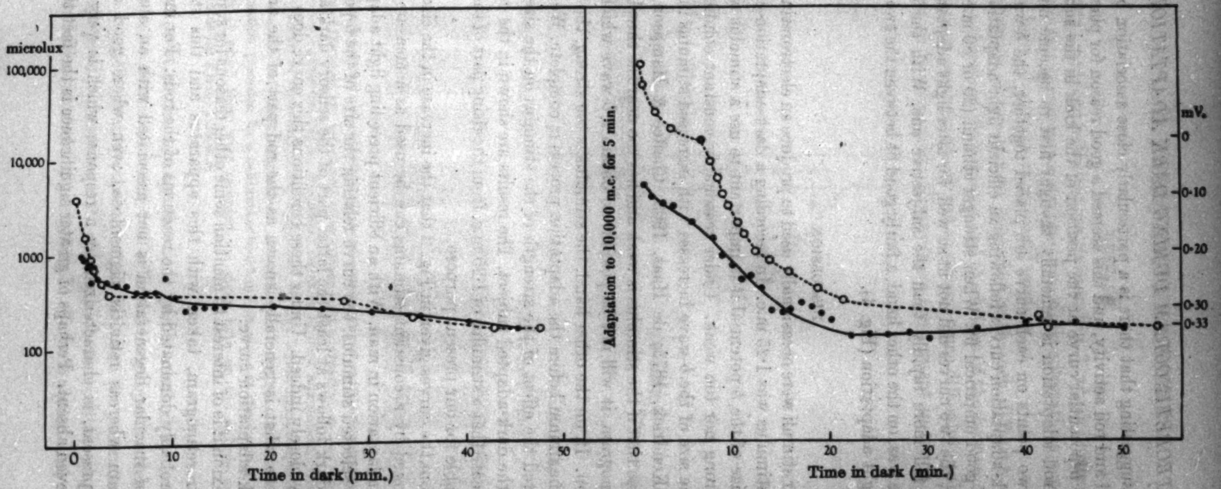

Fig. 4. Effect of a higher stimulus strength on the relation between the *b*-potential dark-adaptation curve and the subjective dark-adaptation curve. Stimulus for E.R.G. 80 m.c. Other details as in Fig. 3.

were unable to evoke any measurable response to a red stimulus (700 mμ) of about this intensity, even though the flash was clearly seen and easily recognized as being red. It is not, therefore, very surprising to find that, during dark adaptation, the electroretinogram only becomes measurable at a time when

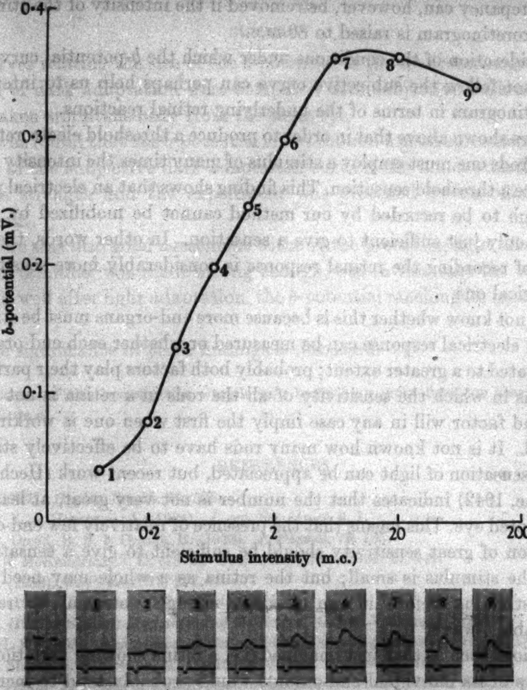

Fig. 5. The effect of stimulus strength on the size of the *b*-potential of the dark-adapted eye. The first E.R.G. was recorded after complete dark adaptation for 40 min.; the intensity of the stimulus was changed by means of neutral tint filters; no stimulus was applied until the eye had recovered its dark-adapted subjective sensitivity. Abscissae: log stimulus intensity. Ordinates: *b*-potential in mV. The E.R.G.'s recorded for each stimulus are given at the bottom of the figure preceded by the calibration to 0·5 mV.

there is at least a likelihood that the rods are beginning to react and that, thereafter, the increase in the *b*-wave follows a curve which can also represent the increase in rod sensitivity.

Difficulties in interpreting the relationship between the *b*-potential and the sensory curves arise, however, when one comes to consider those taken at the beginning of an experiment and not preceded by any special light adaptation. Under these conditions the *b*-potential curve does not follow the rod part of the sensory dark-adaptation curve; a final threshold for the latter may be reached before a maximum *b*-wave can be recorded on the electroretinogram. This discrepancy can, however, be removed if the intensity of the stimulus for the electroretinogram is raised to 80 m.c.

A consideration of the conditions under which the *b*-potential curve follows or does not follow the subjective curve can perhaps help us to interpret the electroretinogram in terms of the underlying retinal reactions.

We have shown above that in order to produce a threshold electroretinogram from the rods one must employ a stimulus of many times the intensity required to produce a threshold sensation. This finding shows that an electrical potential big enough to be recorded by our method cannot be mobilized by a retinal response only just sufficient to give a sensation. In other words, the sensory method of recording the retinal response is considerably more sensitive than the electrical one.

We do not know whether this is because more end-organs must be stimulated before an electrical response can be measured or whether each end-organ must be stimulated to a greater extent; probably both factors play their part. Under conditions in which the sensitivity of all the rods in a retina is not identical the second factor will in any case imply the first when one is working at the threshold. It is not known how many rods have to be effectively stimulated before a sensation of light can be appreciated, but recent work (Hecht, Shlaer & Pirenne, 1942) indicates that the number is not very great, at least in the dark-adapted eye. This means that the presence of relatively few end-organs in a condition of great sensitivity should be sufficient to give a sensation even though the stimulus is small; but the retina as a whole may need a much greater stimulus before it can produce enough potential to register a recognizable *b*-wave.

Conditions such as that just imagined (i.e. a retina showing variations in the sensitivity of its individual end-organs) would probably tend to increase the difference between the sensitiveness of the sensory threshold and that of the electrical method as a measure of the actual retinal response. Now a drastic light adaptation, such as the one we used, will have the effect (and, indeed, this is one of the reasons for its use) of making the initial state of all the rods as uniform as possible and should, therefore, decrease the difference in sensitiveness of the two methods. The same effect would also be achieved by increasing the strength of the stimulus used for producing the electroretinograms, since this would bring in rods too insensitive to play a part when a weaker stimulus is used.

Theoretically, a more satisfactory comparison between the subjective and the E.R.G. curves would be obtained if the latter represented the stimulus intensity necessary just to evoke a recognizable *b*-potential at different times during dark adaptation. We attempted to make such a curve, but found that the difficulty of determining the moment at which such a *b*-potential first appeared rendered the method too inaccurate to be practicable with our apparatus.

SUMMARY

1. Curves relating the size of the *b*-potential of the electroretinogram to the duration of dark adaptation were compared with subjective dark-adaptation curves taken simultaneously from the same eye.

2. It was found that the *b*-potential curve showed a good agreement with the rod part of the subjective dark-adaptation curve if the E.R.G. stimulus was of moderate strength and the experiment was preceded by an efficient light adaptation.

3. There was also good agreement without the preceding light adaptation if the E.R.G. stimulus was made strong enough, but in this case the two curves did not fit well after light adaptation, the *b*-potential reaching its maximum too soon.

4. The significance of these findings is discussed.

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THE MECHANISM OF HAEMOSTASIS IN PERIPHERAL VESSELS

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As early as 1897, Bier called attention to the importance of activity of the severed blood vessels in haemostasis. Küttner & Baruch (1920) described segmental spasm in the traumatized large artery, in consequence of which the injured free end became pulseless. Magnus (1923) reported that in the amputated limb the free ends of the large arteries became completely constricted and did not leak blood when the ligature was removed from the proximal stump. Stegemann (1924*a*, *b*), using the pancreas and mesentery of the living rabbit for observation, concluded that clotting played only a subordinate role in the spontaneous cessation of haemorrhage. Tannenberg & Herrmann (1927) thought that the retraction and contraction of the vessel wall were the chief factors in the spontaneous stoppage of bleeding caused by cutting the vessel. Chen & Tsai (1944) concluded from a series of experiments on the arteries of the rabbit's ear, brain and mesentery that the haemostasis of arterial haemorrhage consisted of two phases, an initial constriction phase and a subsequent coagulation phase. They pointed out that, without the initial constriction, the arrest of bleeding by coagulation was almost impossible because of the high pressure in the artery.

As to the haemostasis in the capillary, most authors think that constriction is the main factor. By means of micro-incision, Magnus (1924) found that the capillaries in the frog's foot and in human skin disappeared completely and remained invisible for a considerable time after cutting. He concluded that capillary haemorrhage was checked by the activity of the capillary itself. Heimberger (1926) observed contraction of human skin capillaries in response to blunt pressure and reported that occlusion may last for a day. He believed this reaction to be due to the contraction of the pericytes. MacFarlane (1941) studied the response of the capillaries of the human finger-nail bed, and revealed that the capillary loop underwent constriction in response to puncturing.

When investigating haemostasis in veins, Hayem (1882) observed the formation of a mass of platelets in the incised jugular vein of the dog and believed it to be important in checking the bleeding. Recently Zucker (1947) re-emphasized the significance of the formation of a platelet plug in venous haemostasis. She described the phenomenon in the venules of the meso-appendix and large branches of mesenteric veins of rats as follows: 'Within one minute a colorless refractile body about 6 μ. in diameter could be seen at the tip of the bleeding stump, but not within the lumen. Subsequent experiment demonstrated the body was formed of platelets.'

METHODS

For the study of arterial haemostasis the vessels of the intact ear and of the exposed brain and mesentery of the rabbit and those of the frog's web and mesentery were used. In the experiments with brain and mesenteric arteries of the rabbit the animals were anaesthetized with chloral hydrate (0·6 g./kg./rectum). The vessels were observed under a low-power microscope (× 48 or 80). A small cutting needle, held by a fine clamp and moved by ball-socket joints, was used for puncturing. The needle was replaced by a fine glass rod with a blunt end when pressure was to be applied. The arteries and arterioles chosen for this study were mostly 0·05–0·5 mm. in diameter.

In order to determine whether the constriction was neurogenic or myogenic in origin, the ear vessels were denervated under local narcosis (procain) by extirpating the superior cervical ganglion and resecting the great and dorsal auricular nerves according to the method of LeCompte (1941). The reaction of the vessels to mechanical injury was tested 2–3 weeks after the operation. Complete degeneration was verified by intra-vitam staining with methylene blue according to the method of Clark, Clark & Williams (1934).

The effect of hypocoagulability of blood was studied in rabbits by giving dicumarol (20 mg./kg./day for 4 days) orally or heparin (880 units/kg.) by intravenous injection prior to observation.

Similar methods were employed for the study of the marginal ear vein of the rabbit and mesenteric veins of the rabbit and frog. In many cases it was necessary to wash the blood off the wound. This was done by applying a tiny stream of warm saline solution.

For experiments with capillaries, we used anaesthetized rabbits, spinal frogs and toads, and toads or frogs whose spinal cords had been previously completely destroyed. Capillaries of 7–35 μ. in diameter were chosen. They were observed under the microscope with a magnification of 480 and punctured by a fine needle with a tip of about 3–5 μ. in diameter. A fine glass rod was employed for applying pressure. The needle or the rod was carried by a small clamp and operated through a series of ball-socket joints.

RESULTS

Arterial haemostasis

Puncture of the rabbit's ear at any spot by a sharp cutting needle may cause a transient blanching of the whole ear lasting 10–30 sec. This is purely a reflex phenomenon since it did not occur in the denervated ear.

Hard pressure on the central artery or on any of its branches by a fine glass rod caused a local constriction lasting 2–6 min. (Pl. 1, fig. 1). Puncture of the artery with a fine cutting needle brought forth a similar but stronger local constriction. The segment disappeared from sight within 30–90 sec. of the injury (Pl. 1, fig. 2). As a consequence of this strong local constriction, bleeding usually stopped spontaneously within 1·5 min. The contracted vessel began to relax 8–15 min. later, but did not regain its former size until 10–20 min. afterwards. The blood was usually washed off by a fine stream of warm saline, but if the clot was allowed to remain on the spot, the constriction usually persisted longer, sometimes for nearly an hour. The constriction usually spread for 0·2–3·3 mm. in both directions from the point of injury.

Occasionally, dilatation supervened for a time during the period of constriction and the vessel appeared to undergo some rhythmic contraction which was again followed by uninterrupted constriction. If the transient dilatation occurred within 2·5–3 min. after the injury, momentary bleeding again ensued. Nevertheless, if it took place after the clot was already very firm, no bleeding was noticed.

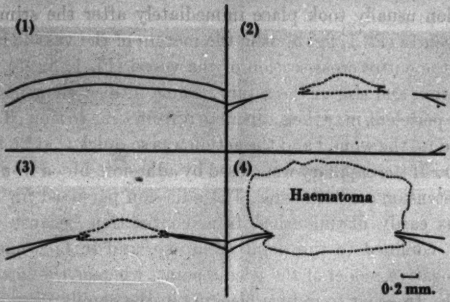
The above-described local response of the artery to mechanical stimulus also occurred in the denervated artery with no apparent delay in onset, no shortening in the duration of response and no diminution in the intensity of constriction. Since the vessels studied were all found by intra-vitam staining with methylene blue to be devoid of nerves it was concluded that the local response of the artery must be myogenic in origin.

To ascertain the relationship between blood coagulation and haemostasis in an artery, five heparinized and three dicumarolized rabbits were employed. Despite the fact that the coagulation time in the heparinized rabbits was increased to between 30 and 120 min., no serious and prolonged bleeding occurred in the artery after puncturing. On the other hand, in the dicumarolized rabbits whose blood was shown to be incoagulable for 2 hr., bleeding was so severe that it inevitably caused the death of the animal. In these animals a puncture of the artery invariably evoked a strong local constriction as in the other rabbits, which checked the immediate bleeding. However, as soon as the constriction passed off, bleeding again took place (Text-fig. 1). This continued till death, if no extraneous measures were taken to prevent it.

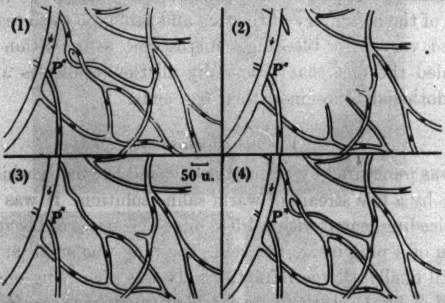
The mesenteric and brain arteries of the rabbit behaved exactly like those in the ear.

Capillary haemostasis

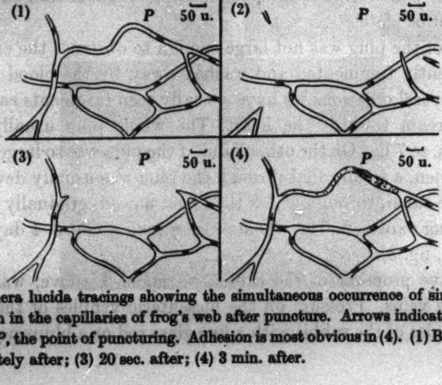
The question of capillary contractility is still in dispute. According to our own experience it differs according to species and situation. In the frog's web the capillary is contractile, whereas in the mesentery of the frog and rabbit it is not. In the former, the contraction in response to mechanical pressure or puncture usually involved a segment from 140 to 500 μ. in length (Text-fig. 2). But this was never observed in the mesenteric capillaries of the rabbit, toad and frog. In these non-contractile capillaries it was observed that parts of the capillary wall adhered to each other at the point of pressure or puncture. In the contractile capillaries adhesion also occurred but could not be seen clearly until the constriction phase waned (Text-fig. 3).



Text-fig. 1. Camera lucida tracings of the ear arteries of a dicumarol-fed rabbit after puncture. (1) Before puncturing; (2) 30 sec. after; (3) 15 min. after; (4) 18 min. after.



Text-fig. 2. Camera lucida tracings showing simple constriction of the frog's web capillaries after light pressure. Arrows indicate the direction of blood flow; *P*, point of pressing. (1) Before pressing; (2) immediately after; (3) 15 sec. after; (4) 30 sec. after.



Text-fig. 3. Camera lucida tracings showing the simultaneous occurrence of simple constriction and adhesion in the capillaries of frog's web after puncture. Arrows indicate the direction of blood flow; *P*, the point of puncturing. Adhesion is most obvious in (4). (1) Before puncturing; (2) immediately after; (3) 20 sec. after; (4) 3 min. after.

The adhesion usually took place immediately after the stimulus. It may occur at the centre (Pl. 1, fig. 3) or in the margin of the vessel (Pl. 1, fig. 4), or may occupy the entire cross-section of the vessel (Pl. 1, fig. 5) in accordance with the location and size of the injury. Some formed elements of the blood, especially the platelets, may then adhere to it from time to time. The adhesion of the wall sealed up the wound and the action was so quick that no bleeding could possibly occur. If the capillary was closed by adhesion, blood would accumulate and cause distension on both sides. The adhesion persisted for several hours.

Adhesion is easily distinguished from contraction because the former is strictly local, confined to the point of injury, and in fact not infrequently produces a negative model of the needle point, whereas the latter spreads for some distance. In order to induce bleeding in the capillaries it was necessary not to withdraw the needle but to tilt it so as to separate the walls. If bleeding occurred, it usually stopped within 30–60 sec.

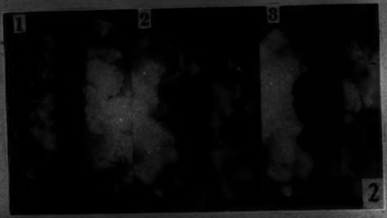
Stretching of the mesentery of the frog and rabbit so as to tease some of the vessels caused very little bleeding. Microscopic examination of the teased vessels revealed the fact that practically all the capillaries and veins were closed by endothelial adhesion at their free ends.

Venous haemostasis

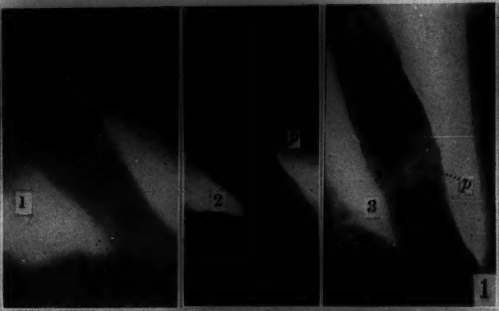
The vein was punctured by a round needle and the blood which exuded was washed away by a fine stream of warm saline solution. It was found that, in most cases, bleeding ceased within 1·5–3 min. The arrest of bleeding was due to the formation of a plug inside the vein. The plug was small at first and then enlarged, until finally a large refractile body was seen lying on the surface of the wound (Pl. 2, fig. 1). If the mass outside the vein was carefully removed by micro-dissection and washing as soon as it was formed, bleeding stopped as usual, i.e. within 1·5–3 min. This was due to the plug found within the vein just below the point of puncture. Microscopic examination showed that the plug consisted of platelets.

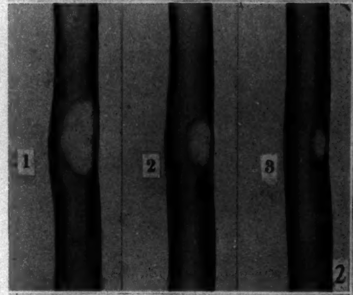
If the size of the plug was not large enough to obstruct the entire lumen, it was subsequently fragmented and washed away by the blood stream (Pl. 2, fig. 2). On several occasions we have actually seen fragments carried away by the blood stream towards the heart. The whole plug usually disappeared within 25 min. to 7 hr. On the other hand, if the plug was so large as to obstruct the entire lumen, a small canal through the plug was usually developed within 24–36 hr. The canal, through which the blood flowed, gradually enlarged until finally the plug vanished. The whole process took about 3–4 days for its completion (Pl. 2, fig. 3).

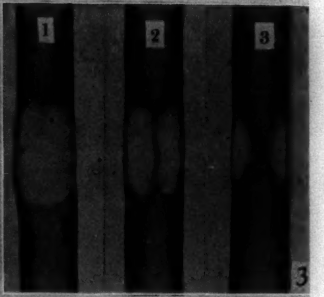
The adhesive property of the capillary, reported above, was also demonstrated in the veins. Pressure on the mesenteric venules of the frog and the auricular venules of the rabbit with a fine glass rod or a blunt needle produced



Figs. 1-5.







Figs. 1–5.

adhesion of the walls (Pl. 2, fig. 4). The venule can be distinguished from the capillary by its thicker wall. It is a common experience that cutting the frog's skin with a pair of scissors usually causes very little or no bleeding. Careful examination of the cut stumps of the veins disclosed the fact that the cut ends were sealed by the adhesion of the vessel walls. Pl. 2, fig. 5 shows closure by adhesion at both cut ends of a branch of the mesenteric vein of the frog. There was very little bleeding after cutting.

DISCUSSION

From the observations described above it is evident that arterial haemostasis consists of two phases, an initial constriction and a permanent coagulation. Without the initial constriction of the vessel the formation of a firm clot, strong enough to support the pressure in the artery, would be difficult or even impossible.

The constriction may be analysed into three components. The reflexogenic component is immediate and lasts for 30 sec. at most. It is usually followed by dilatation. It plays only a small part in haemostasis since it is too brief for the formation of a firm clot. The reflexogenic constriction overlaps the initial part of the myogenic constriction. However, the latter outlasts the former for a considerable period and does not vanish until some time after the clot has been well formed. If bleeding occurs and the clot is not washed away from the wound, the constriction may persist much longer; in several cases it lasted nearly an hour. The prolonged constriction in these cases cannot be merely due to the reaction evoked by mechanical stimulation because mere pressing or puncturing the vessel without allowing the clot to remain on the spot induces constriction with much shorter duration. According to the work of Janeway, Richardson & Park (1918), Tsai, McBride & Zucker (1944) and others, the platelets contain a very potent vaso-constrictor substance which is liberated into the serum when the blood coagulates. It is, therefore, conceivable that the later phase of prolonged constriction as a result of clotting may be due to the action of the vaso-constrictor substance liberated from the disintegrated platelets.

The importance of constriction in arterial haemostasis is clear from the second bleeding which occurs in the dicumarolized animal after the constriction has passed off. In other words, the constriction phase, though it cannot stop bleeding permanently, is necessary for the formation of a firm clot.

The absence of the second bleeding in the heparinized rabbit is difficult to explain. It may be due to neutralization of heparin by cephalin at the site of injury, but this is merely a conjecture.

Previous workers all considered capillary contraction as the chief mechanism of capillary haemostasis (Magnus, 1924; MacFarlane, 1941; and others). But according to our present investigation, emphasis must be laid on the significance of the adhesive property of the capillary endothelium. In the first place, contraction of the capillary does not always follow mechanical stimulation or injury. For instance, in the frog's web some capillaries do contract in response to mechanical stimuli, but the contraction never lasts more than 30 sec., while many others do not contract at all. Furthermore, in the mesentery of the frog and rabbit none of the capillaries shows any contractility after mechanical stimulation. On the other hand, adhesion of the capillary wall is the most constant reaction observed. It occurs at the point of puncture or pressure and persists for as long as an hour, in which period the walls rarely separate spontaneously. In the contractile capillaries the walls adhere together in the same way, but this adhesion cannot be seen clearly until the contraction passes off. From this we may conclude that the chief mechanism of capillary haemostasis is the adhesion of the vessel's walls, contraction being unimportant. The adhesive property of the capillary endothelium is manifested only when the cells are injured and pressed against each other. This assumption is deemed necessary, otherwise one would have to demonstrate adhesion after constriction, which however never happens.

As the phenomenon of capillary adhesion after mechanical injury is so constant and persistent, it is difficult to explain why it has escaped the attention of previous workers, unless it was mistaken for capillary contraction. According to our own experience, capillary contraction and adhesion are two distinct phenomena easily distinguished from one another under our experimental conditions. In the first place, adhesion is local and persistent, while contraction spreads along the vessel and is transient.

We agree with Zucker (1947) that the formation of a platelet plug is the chief mechanism in the venous haemostasis. But we wish to add that in the venules the adhesion of walls as a result of injury is also important in stopping bleeding. The endothelium of the large vein may also possess this property, but, because of its wider lumen, a small point of adhesion can easily be broken through and the walls again separated by the flow of blood. Therefore, in large veins adhesion plays no essential role in haemostasis.

It should be pointed out that most of the veins are responsive to mechanical stimulation and injury. But the constriction is too mild and too brief to have any value in haemostasis.

The formation of a platelet plug in the injured vein is exceedingly interesting in view of its relation to haemostasis and thrombosis. It is well known that the platelet possesses an adhesive property and will attach to any rough surface with which it comes into contact. On account of the more rapid flow and higher pressure in the artery any platelet adhering to the damaged lining would tend to be washed away by the blood stream, thus leaving no opportunity for the platelets to accumulate and form a plug on the spot. On the other hand, the slow stream in the vein favours the adhesion and accumulation of platelets at the point of injury.

The mechanism of fragmentation and recanalization of the platelet plug is not clear. It may be due to the disintegration of the platelets or fibrinolytic action of the serum tryptase. Whether recanalization is due to the invasion of endothelial growth has not been studied in the present investigation.

The adhesive property of the capillary and venule may be an important factor in determining bleeding time. Its loss may result in the prolongation of bleeding time without affecting coagulation time. The clinical bearing of this point should be investigated.

SUMMARY

1. Arterial haemostasis consists of two phases, constriction and coagulation. The constriction evoked by mechanical injury may be composed of three elements: (1) reflex constriction, (2) local muscular response, and (3) response to chemical substances liberated from the disintegrated platelets. The local muscular response which is the most important is sufficient to stop bleeding till the formation of a firm clot. Reaction to vaso-constrictor substances would come later. Permanent arrest of haemorrhage depends upon the formation of a firm clot but the retention of a firm clot in place would be impossible without the initial constriction.

2. The mechanism of capillary haemostasis is mainly adhesion of the capillary wall as a consequence of injury of the endothelium. Adhesion causes a persistent closure of the vessel. Capillary contractility may be present in some capillaries such as those in frog's web, but it is insignificant in haemostasis because of its inconsistency and brief duration.

3. The formation of a platelet plug and adhesion are the two important mechanisms for haemostasis in veins but adhesion in large veins is rarely effective in stopping haemorrhage. Venous constriction plays a minor role in haemostasis.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Photographs showing constriction of a rabbit's ear artery after heavy pressure by a blunt needle. ×18. (1) Before pressing; (2) immediately after; (3) 1·5 min. after; (4) 4·5 min. after.

Fig. 2. Photographs showing constriction of a rabbit's ear artery after puncture. ×18. (1) Before puncturing; (2) 1 min. after; (3) 8 min. after.

Fig. 3. A photograph showing central adhesion in a capillary of toad's mesentery produced by a sharp puncture. ×200.

Fig. 4. A photograph showing marginal adhesion in a capillary of toad's mesentery produced by a sharp puncture. ×200.

Fig. 5. Photograph showing complete obstruction by adhesions in a capillary of toad's mesentery produced by several punctures. ×200.

Plate 2

Fig. 1. Photographs showing the formation of platelet plug in a rabbit's ear vein after a sharp puncture. *p* indicates the platelet plug. ×18. (1) Before puncturing; (2) 2 min. after; (3) 7 min. after.

Fig. 2. Diagrams showing fragmentation of platelet plug in the marginal ear vein of a rabbit. (1) 7 min. after puncturing; (2) 15 min. after; (3) 23 min. after.

Fig. 3. Diagrams showing recanalization of platelet plug in the marginal ear vein of a rabbit. (1) 7 min. after puncturing; (2) 24 hr. after; (3) 48 hr. after.

Fig. 4. Photograph showing adhesion in a vein in frog's mesentery after a sharp puncture. ×30.

Fig. 5. Photograph showing adhesion in a vein in frog's mesentery after cutting by scissors. Note the adhesion at both cut ends. ×30.

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THE ACTION OF ACETYLCHOLINE AND ADRENALINE ON FLEXOR AND EXTENSOR MOVEMENTS EVOKED BY STIMULATION OF THE DESCENDING MOTOR TRACTS

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The study of the action of chemical substances on the central nervous system has been made by many workers using different methods. Naturally, the relations are very complicated and it is not surprising that the results obtained have been contradictory. Recently Calma & Wright (1947) have drawn up a table showing the different observations recorded when eserine has been applied, and this sufficiently illustrates the confusion concerning this substance alone.

One method of simplifying the conditions was described by Bülbring & Burn (1941) who worked on the perfused spinal cord of the lower half of the dog and studied the action of substances on the knee jerk and the flexor reflex. In this preparation, impulses from higher centres were excluded by cutting the cord at D12, and a uniform blood supply was obtained by perfusion from a pump.

Another approach to the study of the transmission processes in the spinal cord has been introduced by Skoglund (1947). This consists in stimulation of the descending tracts in the medulla which, by reversing the direction of the stimulating current, produces either extensor or flexor movements at will. When this is done in cats in which the dorsal roots are cut, the movements are uncomplicated by sensory impulses from the limbs themselves.

Bülbring & Burn (1941) found that eserine and prostigmine increased the flexor reflex, and that acetylcholine, even in the presence of eserine, never depressed it; on the other hand, eserine, prostigmine and acetylcholine in the presence of eserine always depressed the knee jerk (an extensor reflex). Bernhard, Skoglund & Therman (1947) applied acetylcholine and adrenaline to the exposed surface of the lumbar cord and studied the effect on transmission processes at this level when the descending tracts were stimulated. They found that acetylcholine facilitated flexor and depressed extensor responses. Adrenaline usually had the opposite effect to acetylcholine on both systems. The effect of acetylcholine and adrenaline was also studied on the electrotonic cord potentials of opposite sign associated with flexion and extension respectively (cf. Bernhard & Therman, 1947), and on the potential level in the spinal cord recorded from a ventral root; it was found that these substances affected the electrotonic potentials and displaced the potential level in opposite directions.

We have now investigated further flexor and extensor movements of the whole limb and contractions of separate muscles produced by stimulation of the descending motor tracts when injections of acetylcholine and adrenaline were made into the arteries leading to the spinal cord.

METHOD

Decerebrate cats were used. The dorsal roots were cut on both sides from L3 or L4 downwards, including those of the cauda equina. The ventral surface of the medulla was exposed between the base of the skull and the first vertebra. The stimulating electrode was the free point of an enamelled needle placed in a position described by Skoglund (1947) so that flexion or extension could be produced. The predominance of the one or the other depended on slight alterations in the position of the needle and on the condition of the animal. Single square wave stimuli of a duration of about 10 msec. were used at a frequency of 1 per sec.

In some experiments movements of the left leg were recorded. This was done by suspending the leg by the skin over the knee joint and attaching the foot to a light isotonic lever. When the leg was arranged in this way, flexor movements both in the knee joint and in the ankle joint pulled the lever in one direction, while extensor movements at both joints caused it to move in the opposite direction. In these experiments observation was limited either to flexion or to extension.

In other experiments the distal attachments of tibialis anterior and of gastrocnemius were freed and each was attached to a tension lever so that a simultaneous record could be obtained. The strength of stimulus used was just suprathreshold, sufficient to cause a response big enough to be recorded. By increasing the strength of stimulation larger movements could be obtained, but this was not done in order to avoid non-selective muscle effects and general excitation. Intra-arterial injections were made through a cannula tied in the caudal artery or in the right external iliac artery, pointing towards the aorta, the internal iliacs and caudal arteries being ligatured. To ensure that the injection was carried via the spinal arteries to the spinal cord and not to the periphery, clamps were placed temporarily (up to 5 min.) on the arteries leading to the legs. In the majority of experiments the animals were eviscerated. The blood pressure was recorded from a cannula in a carotid artery.

RESULTS

Contrary effects of acetylcholine and adrenaline

Results on transmission processes. A study of these substances was first made on movements of the leg as a whole. Fig. 1 shows one type of reaction to acetylcholine; when 0·2 mg. was injected while flexor responses were recorded (*a*), these were at once increased to more than twice their height. In Fig. 1 (*b*) extensor responses were recorded and the same dose of acetylcholine was repeated. The record shows immediate but short-lasting inhibition of the responses. The opposite effect caused by adrenaline is shown in Fig. 2; when extensor responses were recorded as in (*a*), the injection of 0·2 mg. caused first a change in the base line corresponding to increased extension of the leg, and at the height of extension the responses began to increase in size and remained greater for some minutes. In Fig. 2 (*b*) flexor responses are recorded, and the injection of 0·2 mg. caused a depression, this being again in contrast to the action of acetylcholine.

In Fig. 1 (*b*) the effect of acetylcholine on the extensor twitches was inhibitory but transient. In some experiments the inhibition was of long duration. As an example of this an experiment will be described, a portion of which is illustrated in Fig. 3. In the earlier part of the experiment a study was made of extensor responses in which it was found that repeated doses of acetylcholine depressed them. The original size of responses was restored by successive increases of stimulus strength. Finally, a state of complete block in the cord was reached and no further increase of stimulus strength could break through it. The injection of 0·2 mg. adrenaline, however, restored the extensor responses and caused a large increase in extensor tone. The stimulus of the original strength now sufficed. Evidently the acetylcholine had caused a steadily diminishing excitability which was restored by adrenaline. These effects were repeatable. Thus the injection of the small dose of 20 μg. acetylcholine caused the effect shown in Fig. 3 (*a*), namely, an initial increase in extensor responses and then a prolonged disappearance. Block appeared once more so that even with supramaximal stimuli no extension was obtained. The injection of 50 μg. adrenaline then produced the result seen in Fig. 3 (*b*) and the stimulus strength required returned to the initial value. This removal of block by adrenaline is also seen in Fig. 5, to which reference will be made later.

Fig. 1.

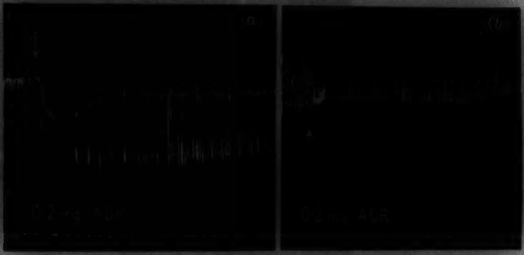

Fig. 2.

Fig. 1. Decerebrate cat. Records of responses in the whole leg to stimulation of the descending tracts in the medulla. (*a*) Facilitation of flexor responses by 0·2 mg. acetylcholine (contractions upwards); (*b*) depression of extensor responses (contractions downwards).

Fig. 2. Records as in Fig. 1. (*a*) Shift of base line to extensor side and facilitation of extensor responses by 0·2 mg. adrenaline; (*b*) depression of flexor responses.

In some experiments, the action of acetylcholine was not immediately to increase flexor responses as shown in Fig. 1 (*a*), but this increase was preceded by a short period of inhibition. In Fig. 4 is a record of the contractions of the anterior tibial muscle (upper tracing). When 0·2 mg. acetylcholine was injected the responses of this flexor muscle were first depressed and then increased. In this experiment the medullary stimulation was arranged in such a way as to elicit responses simultaneously in both the anterior tibial and the gastrocnemius muscle (lower tracing). It is interesting to note that, coinciding with the period of flexor depression, there was extensor augmentation. This reciprocal relation is also shown in Fig. 5 (*a*), in which the changes in the extensor responses were a mirror image of those in the flexor, to the point at which both were blocked. This block was removed by a slow intravenous infusion of adrenaline (Fig. 5 (*b*)).

Fig. 3. Record of responses in the whole leg. (*a*) 20 μg. acetylcholine causing initial facilitation of extensor responses followed by depression; (*b*) 50 μg. adrenaline restoring extensor responses.

In other experiments acetylcholine depressed the flexor responses without much subsequent augmentation (Fig. 9 (*a*)). In these experiments adrenaline produced no effect at all or else caused an increase of flexor movements. Whatever the result of injecting acetylcholine, the effect of injecting adrenaline, provided that the doses were not excessive, was in the reverse direction.

The diphasic action of acetylcholine seen in Figs. 4 and 5 appears also in Fig. 6. Here both systems were activated simultaneously. In the beginning of the experiment, sometimes flexor and sometimes extensor responses were predominant, apparently due to spontaneous fluctuations in the excitability of the cord. As the record is of the responses in the whole leg, and therefore represents the algebraic sum of the activity in both systems, this experiment was particularly suitable to study the effects of substances on the balance between the two systems. When 0·2 mg. acetylcholine was injected (Fig. 6 (*a*)), the immediate effect was a series of large extensor responses followed by a reversal to small flexor responses. After a minute, a third stage followed when the responses became once more extensor. In this experiment the fall in blood pressure produced by acetylcholine was followed by an unusual rise. This suggested the possibility that the third stage might have been produced by a liberation of adrenaline from the cat's suprarenal glands. Both adrenals were therefore removed and the experiment was repeated. The injection of acetylcholine (Fig. 6 (*b*)) now caused the same initial increase of extensor responses followed by a reversal to flexor responses as before. This stage, however, persisted and was no longer followed by extensor responses. The evidence is thus

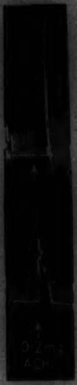

Fig. 4.

Fig. 5.

Fig. 4. Simultaneous activation of flexor and extensor systems recorded separately from the tibialis anterior (upper tracing) and the gastrocnemius (lower tracing). 0·2 mg. acetylcholine caused initial depression of flexor responses followed by augmentation. Coinciding with flexor depression there is extensor augmentation.

Fig. 5. Records as in Fig. 4. (*a*) The diphasic action of 0·2 mg. acetylcholine on flexor responses (upper tracing) and the reciprocal effect on extensor responses (lower tracing) followed by complete depression of both; (*b*) a slow infusion of adrenaline restored the responses, and 0·2 mg. acetylcholine failed to depress them.

strong that these were due to adrenaline liberated from the adrenal gland. In Fig. 6 (*c*) and (*d*) are shown effects corresponding to those in (*a*) and (*b*), but due to a larger dose of 0·5 mg. acetylcholine. The uncomplicated effect of the acetylcholine is again evident in Fig. 6 (*d*) after the removal of the adrenals. In Fig. 6 (*c*) the second stage of flexor responses was almost completely masked by the output of adrenaline, and appeared only as a diminution of the extensor responses. The corresponding effects of these injections on the blood pressure are shown alongside.

Fig. 6. Shows records as in Fig. 1 and corresponding records of blood pressure. (*a*) The effect of 0·2 mg. acetylcholine was first a phase of dominating extensor responses, then small flexor responses; finally extensor responses; (*b*) the third phase was removed by exclusion of the suprarenal glands; (*c*) and (*d*) are the effects of 0·5 mg. acetylcholine before and after removal of the suprarenals. (See text.)

Excitatory effects. One effect shown in Fig. 3 (*a*), to which attention has not been drawn, is the rise in the base line corresponding to increased tension ('tone') in the flexor muscle after the injection of acetylcholine, which is to be contrasted with the increase in extensor tone shown in Fig. 2 (*a*) after the injection of adrenaline. The increase in flexor tone in Fig. 3 (*a*) appears to be independent of the transmission process involved in the responses. This is shown more clearly in Fig. 7 (*a*) where flexor responses were recorded. The injection of 0·4 mg. acetylcholine caused first a small increase in these responses, which were then interrupted by a very large flexion of the leg. To show that movements of flexion and extension of this kind were independent of the activity produced by the medullary stimulation, we recorded the effect of injecting acetylcholine and adrenaline in the absence of stimulation. This is shown in Fig. 7 (*b*). The injection of 0·2 mg. acetylcholine produced a powerful flexor movement which was maintained with variations until the injection of 50 μg. adrenaline. This not only abolished the flexion but produced extension as shown.

Fig. 7. Flexor movements of whole leg (flexion upwards). (*a*) 0·4 mg. acetylcholine caused increased flexor responses to medullary stimulation on which was superimposed the large flexion of the whole leg; (*b*) in absence of stimulation of descending motor tracts, 0·2 mg. acetylcholine caused flexion of leg maintained with variations until 0·05 mg. adrenaline was injected. This abolished flexion and produced extension.

The modification of the action of acetylcholine

Adrenaline. In the previous experiments we have made separate studies of the effects of acetylcholine and adrenaline. Since these two substances are known to affect one another, adrenaline causing potentiation or depression of acetylcholine action (Burn, 1945), we have also paid attention to modification of the action of acetylcholine by adrenaline. An example of this is seen in Fig. 8 which is a record of the responses of the gastrocnemius. In Fig. 8 (*a*) the injection of acetylcholine caused the less usual effect of increased extensor responses; a slow intravenous infusion of adrenaline was then begun and, during this infusion, the response to acetylcholine was a depression of extensor responses (Fig. 8 (*b*), (*c*)). This depression is the more usual effect seen in Figs. 1 (*b*), 3 and 6.

A simple potentiation of the inhibition of extensor responses by acetylcholine was also observed. In Fig. 3 (*a*) a depression of extensor responses was produced by the injection of 20 μg. acetylcholine. Just before 0·2 mg. adrenaline had been injected and it seemed that this was responsible for the large effect of so small a dose as 20 μg. Earlier a less pronounced effect of acetylcholine was produced by 0·4 mg.

In the experiment shown in Fig. 5 the initial effect produced by acetylcholine was inhibition of flexor responses. During a slow intravenous infusion of adrenaline this initial inhibition was no longer seen, nor did the acetylcholine cause a subsequent block.

In one experiment adrenaline also modified the excitatory effects of acetylcholine. At first acetylcholine caused a general extension of the leg, but after adrenaline it caused a flexion similar to that in Fig. 7.

Fig. 8.

Fig. 9.

Fig. 8. Record of gastrocnemius. (*a*) 0·2 mg. acetylcholine caused increased extensor responses. During adrenaline infusion, the same dose caused inhibition (*b*), seen more obviously in (*c*) after 0·5 mg.

Fig. 9. Records as in Fig. 4. (*a*) 0·2 mg. acetylcholine caused inhibition of flexor movements with simultaneous increase of extensor; (*b*) after 0·05 mg. eserine, the same dose of acetylcholine increased the flexor movements and depressed the extensor.

Eserine. The injection of eserine had no immediate effect, though it produced a gradual depression of all responses, the flexor responses disappearing earlier than the extensor. In one experiment only the injection of 1 μg. and later of 10 μg. eserine caused an immediate transitory depression.

The remarkable feature of eserine was the reversal which it produced in the effect of acetylcholine: Fig. 9 shows an experiment in which flexor and extensor responses were produced simultaneously and recorded separately. The injection of 0·2 mg. acetylcholine caused a depression and a slight subsequent increase of the anterior tibial responses and an increase in the gastrocnemius responses (Fig. 9 (*a*)). When 50 μg. eserine was given, the repetition of the acetylcholine injection resulted in changes which were the reverse of those obtained previously. The flexor responses were increased and the extensor responses were depressed (Fig. 9 (*b*)).

DISCUSSION

The methods used in these experiments call for some attention in the first place. The method of stimulating the descending motor tracts in deafferentated animals makes it possible to produce flexor or extensor responses uncomplicated by changes in afferent inflow (though there remains the possibility of backfiring along motor fibres (Lloyd, 1942)). This central activation of the motor system, however, involves complex excitatory and inhibitory processes at the lower levels of the spinal cord. Thus the opposite effect of one drug on flexor and extensor responses, especially when the two systems are activated simultaneously, may be due to the nervous connexions involved in reciprocal innervation. Although many interpretations are possible, we think it is worth while to compare the excitability changes of both flexor and extensor systems under the influence of different substances. It is possible to arrange our own observations in some order as shown in Table 1.

TABLE 1

	Results in 11 experiments		Results in 6 experiments	
	On flexion	On extension	On flexion	On extension
Acetylcholine	(−)+	(+)−	−(+)	+
Adrenaline	−	+	+ or 0	− or 0

+ Indicates increase of transmitted responses; − indicates depression of transmitted responses; a sign in brackets indicates a transient effect preceding or following the main effect; 0 means no effect.

The main effect of acetylcholine may be either increase or decrease of flexion, but whatever its action, the effect on extension is the opposite. Further, the effect of adrenaline is opposite to that of acetylcholine whichever system is studied.

It is, however, unsatisfactory merely to record that some experiments gave results in one direction while others gave results in the opposite direction, and a suggestion is required to harmonize the whole. The action of acetylcholine at the neuromuscular junction and in the sympathetic synapses is known to be diphasic, producing both stimulation and paralysis, and it is therefore not surprising to find a diphasic action in the central nervous system. In some conditions extension is facilitated while in others it is depressed; further the two effects may follow one another in one observation. A similar diphasic action occurs when flexion is recorded.

Why one phase of this diphasic action dominates in one experiment whereas another phase dominates in a second can be explained in different ways. It may be due to the initial state of the cord; inevitable variations in operative procedure, variations in circulation, may all intensify initial differences in the biochemical milieu of the spinal cord of different animals. The conditions under which a particular type of response is obtained remain to be determined by further investigation. We know already one factor which changes the type of response, namely, the amount of adrenaline present. Table 1 shows that, so far as single injections are concerned, adrenaline produced the opposite response to acetylcholine, but we also found that, during the period after an injection of adrenaline or during a slow intravenous infusion, the action of acetylcholine was modified. Thus, when in the beginning of an experiment acetylcholine increased the extensor responses, later, in the presence of adrenaline, it depressed them. Thus a reaction of the type described on the right of Table 1 was changed to a reaction described on the left. Further, when the administration of successive doses of acetylcholine had produced a condition in which it was no longer possible to elicit muscular responses to medullary stimulation, the cord being blocked, the injection or infusion of adrenaline restored the transmission. Such results appear to indicate that there is not only a simple antagonism between the two substances as shown in Table 1, but that the action of acetylcholine depends on the amount of adrenaline present.

SUMMARY

1. The effect of acetylcholine and adrenaline has been studied separately on extensor and flexor responses produced by stimulation of the descending tracts in the medulla. The observations were made in decerebrate cats in which the sensory roots were divided. The substances were injected intra-arterially so as to pass through the spinal arteries to the cord.

2. The effect of acetylcholine was observed as: (*a*) a simple increase in extensor responses; (*b*) a simple depression of extensor responses or one which is preceded by a transient phase of facilitation; (*c*) a simple increase of flexor responses or one which is preceded by a transient phase of depression; (*d*) a depression of flexor responses. The effects (*b*) and (*c*) were commonly observed; the effects (*a*) and (*d*) were rare.

3. The effect of a single injection of adrenaline was observed as: (*a*) no effect at all or a simple depression of extensor responses when the effect of acetylcholine was as in 2 (*a*); (*b*) an increase of the extensor responses (corresponding to 2 (*b*)); (*c*) a depression of flexor responses (corresponding to 2 (*c*)); (*d*) an increase of flexor responses or no effect at all (corresponding to 2 (*d*)); (*e*) a restoration of extensor and flexor responses when these had been blocked by acetylcholine.

4. Slow intravenous infusion of adrenaline modified the action of acetylcholine so that (*a*) facilitation of extensor response was changed to depression of extensor response; (*b*) the same depression of extensor responses was produced

by a much smaller dose of acetylcholine; (*c*) a depression of flexor responses by acetylcholine was no longer seen.

5. It is possible to produce flexion by the intra-arterial injection of acetylcholine in the absence of medullary stimulation. This flexion is abolished by adrenaline, which produces extension.

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THE EFFECTS OF SMALL AMOUNTS OF CARBOXYHAEMOGLOBIN ON THE DETERMINATION OF OXYGEN IN THE BLOOD BY THE HALDANE AND VAN SLYKE METHODS

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It has been shown by Courtice & Douglas (1947) that if a borate buffer of pH 10 is used instead of sodium carbonate solution, the Haldane method of blood-gas analysis gives values for the percentage oxygen capacity which are practically identical with those obtained by the van Slyke manometric method. In their experiments, any discrepancies between the results obtained by these two methods due to the presence of carboxyhaemoglobin were minimized by using blood from the ox, sheep and rabbit (subsequent analysis of blood from these animals in Oxford showed absence of CO), and human blood taken from non-smokers or from smokers at least 12 hr. after smoking.

It is well known that as much as 2 vol. % of carbon monoxide may be found in the blood of smokers during the course of a day (cf. Roughton, Darling & Root, 1944). If CO is quantitatively liberated in the Haldane apparatus, then the determined oxygen capacity of a given sample of blood by this method should equal the oxygen capacity by the van Slyke method plus the CO content. This has been investigated experimentally together with the variability of CO content of the blood of smokers and non-smokers.

METHODS

The apparatus and experimental procedure for determining the oxygen capacity by the Haldane method have been described by Courtice & Douglas, and no change has been made in the present investigations. Oxygen capacities of 1 ml. samples of the blood, previously saturated with air, have been determined with the van Slyke manometric apparatus by the usual technique. The coefficients of solubility of oxygen used in the calculation by this method were those of Sendroy, Dillon & van Slyke (1934).

For concurrent determinations of oxygen capacity by both methods, approximately 10 ml. of venous blood were withdrawn and put into a 100 ml. Erlenmeyer flask containing dry potassium oxalate as an anticoagulant. The flask was stoppered and rotated manually for not less than 20 min. in a water-bath, the temperature of which was measured to determine the amount of oxygen in solution. This flask was employed in preference to a tonometer because of possible drainage errors in sampling from the latter (Roughton *et al.* 1944; Courtice & Douglas, unpublished observations).

That the blood was in fact fully saturated was checked by repeating the van Slyke determinations after a further period of shaking.

The CO content of blood was determined by the Scholander-Roughton syringe-capillary apparatus employing the special method for blood containing small amounts of CO (Roughton & Scholander, 1943). Where the determinations were employed in conjunction with the Haldane and van Slyke methods of determining oxygen capacity, the CO content of the sample was determined after saturation with air. When variations in CO content were investigated, venous blood was stored in syringes on ice in the dark as recommended by Scholander & Roughton. It was found, in agreement with Roughton *et al.*, that changes in CO content of a stoppered sample of blood, even after equilibrating with air, were slight where any difference was detectable.

All analyses by all methods were performed in duplicate. The CO determinations were all done by the same individual (W. J. S.), but the van Slyke and Haldane determinations were done at random by both authors, the same individual rarely doing both these determinations on the same sample of blood. For both van Slyke and Haldane methods the mean difference between duplicates was 0·09 vol. % and the standard deviation of the differences 0·08 vol. %. For the CO contents the mean difference between duplicates was 0·04 vol. % and the standard deviation of the differences 0·03 vol. %.

RESULTS

The oxygen capacity by Haldane method compared with the oxygen capacity by van Slyke method plus CO content of blood

The results are summarized in Table 1. The individual values represent the mean of duplicate determinations. The mean difference between oxygen capacity (van Slyke) plus CO content and oxygen capacity (Haldane) is 0·02 vol. % and the standard deviation of the differences is 0·10 vol. %. It may be concluded, therefore, that the Haldane method quantitatively liberates CO present in the blood up to 2 vol. % at least. Usually, gas liberation was complete within 5–7 min. after adding the ferricyanide, with steady volume readings confirmed during shaking for a further 5–10 min. With the higher CO contents, slightly longer periods of shaking, e.g. 9–10 min., were sometimes observed before constant volume readings were obtained.

TABLE 1. Oxygen capacity of human whole blood by Haldane and van Slyke methods, and CO content by Scholander-Roughton method. All values ml./100 ml. blood

	O_2 capacity van Slyke	CO content	CO content + O_2 van Slyke	O_2 capacity Haldane
	21·4	0·1	21·5	21·5
	19·7	0·1	19·8	19·6
	21·4	0·1	21·5	21·8
	21·3	0·3	21·6	21·7
	19·1	0·3	19·4	19·2
	18·7	0·3	19·0	19·1
	20·1	0·3	20·4	20·4
	18·7	0·4	19·1	19·0
	20·2	0·4	20·6	20·7
	18·5	0·6	19·1	19·0
	19·7	0·7	20·4	20·2
	19·6	0·7	20·3	20·3
	20·0	1·0	21·0	21·1
	19·1	1·2	20·3	20·2
	17·7	1·4	19·1	19·1
	17·7	2·6	20·3	20·2
Mean	19·56	0·66	20·22	20·20

There has been considerable discussion recently concerning the validity of the oxygen capacity, as usually determined, as a measure of the oxygen capacity of the circulating blood. van Slyke, Hiller, Weisiger & Cruz (1946) present evidence for the presence in circulating blood of an inactive, probably ferric, variant of haemoglobin which gradually reverts to the active form when drawn blood is allowed to stand. They also review the literature on the subject. In the present investigation the oxygen capacity (Haldane method) of a number of blood samples was determined as soon as possible after withdrawal, at hourly intervals up to 8 hr. and then at 24 hr., the blood being stored meanwhile in stoppered test-tubes in the dark on ice. Of ten such samples only one gave a significant increase of 0·4 vol. % after standing for 24 hr., and the mean difference of all ten samples in this time was zero. During the first 8 hr. after drawing the blood there was no significant trend in oxygen capacity. This small series of results merely indicates the reproducibility of the oxygen capacity by the Haldane method when the blood is stored as described. When comparing the oxygen capacity by the Haldane and van Slyke methods, both analyses were carried out either at the same time or within a short interval of one another. It is therefore unlikely that the oxygen capacity of any sample of blood altered significantly by reversion of inactive to active haemoglobin during the course of these analyses.

The effect of smoking on the CO content of the blood

The morning CO content of the blood of smokers and non-smokers. The CO content of the blood of ten smokers and eight non-smokers was determined in the morning about 12 hr. after the first group had last smoked a cigarette. The results are shown in Table 2. Values obtained for different individuals varied from 0·05 to 0·57 vol. % in smokers and from 0·02 to 0·18 vol. % in non-smokers. Repeated determinations were made on five of the smokers during

TABLE 2. CO content of blood of smokers 12 hr. after smoking, and of non-smokers, ml./100 ml.

Smokers		Non-smokers	
Subject	CO content	Subject	CO content
W. W.	0·05	M. M. B.	0·05
F. C. C.	0·20, 0·22, 0·23, 0·24, 0·23, 0·15	B.	0·02
H.	0·25	C. G. M.	0·05
T. J. M.	0·28, 0·23	M. V. B.	0·17
P. J. R. P.	0·28	W. M. J.	0·12
G.	0·42	G. R.	0·14
E. T. G.	0·39, 0·46	R. G. T.	0·18
P. A. L.	0·43	K.	0·16
W. J. S.	0·41, 0·47, 0·53, 0·56		
R. W. G.	0·57, 0·57, 0·53, 0·55, 0·52, 0·54		

a period of about 3 months and were found to be fairly constant in any individual, but in different individuals the level was not related to the number of cigarettes smoked per day. F. C. C. and R. W. G. each smoked about 20 cigarettes per day whereas E. T. G. and W. J. S. smoked only 5–10 per day.

The rise in CO content of the blood after smoking 5 *cigarettes in* 2 *hr*. In these experiments five subjects were used. A sample of blood was taken in the morning, 12 hr. after smoking. Five cigarettes were then smoked in 2 hr. and a further sample of blood withdrawn for CO estimation. The results are given in Table 3. The increase in CO content varied widely in different individuals, but in the same individual repeated determinations on different days gave similar results. Thus in two subjects, F. C. C. and T. J. M., the increase was only 0·2 vol. %, whereas in the other three subjects, W. J. S., E. T. G. and R. W. G., the increase was on the average 0·9 vol. %. Thus in those subjects with the highest initial CO content, the rise in CO as a result of smoking 5 cigarettes in 2 hr. was greatest.

TABLE 3. The effect of smoking 5 cigarettes in 2 hr. on CO content of blood, ml./100 ml.

			W. J. S.		E. T. G.		
	F. C. C.	T. J. M.	(*a*)	(*b*)	(*a*)	(*b*)	R. W. G.
Before smoking	0·23	0·23	0·41	0·53	0·39	0·46	0·52
After smoking	0·45	0·44	1·25	1·43	1·43	1·42	1·42

TABLE 4. CO content of blood of smokers throughout the day, ml./100 ml.

	10 a.m.	12.30 p.m.	4.30 p.m.
T. J. M.	0·19	0·23	0·27
F. C. C.	0·32	0·44	0·41
F. C. C.	0·48	0·62	0·53
W. J. S.	1·08	1·06	1·29
R. W. G.	1·02	1·62	1·29

CO content of the blood of smokers throughout the day. In four subjects, the CO content of the blood was determined at 10 a.m., 12.30 p.m. and 4.30 p.m., during the course of their normal laboratory duties. The results are shown in Table 4. Here again the CO content of the blood throughout the course of the day does not run parallel with the number of cigarettes smoked, for F. C. C. and R. W. G. each smoked about three times the number of cigarettes smoked by W. J. S. and T. J. M. The amount of CO absorbed must therefore depend on the manner in which the cigarettes are smoked rather than on the number, even though all the smokers in this series inhaled. That the absorption of CO from the lungs or the affinity of haemoglobin for CO did not differ significantly was investigated in two subjects, F. C. C. who showed a small increase in blood CO on smoking and W. J. S. who showed a large increase. A mixture of air containing 1% CO was made up in a Douglas bag. The subjects sitting at rest took every fifth inspiration from this bag for 100 breaths. From the rise in CO content of the blood and the blood volume of each subject, the actual amount of absorbed CO was calculated. This was found to be approximately the same in both individuals.

DISCUSSION

The experiments on oxygen capacity of blood containing appreciable amounts of carboxyhaemoglobin provide a further test of the validity of the Haldane method as modified by Courtice & Douglas (1947). At the outset it was not certain that the CO would be liberated quickly enough by this method, as the CO from fully saturated blood is liberated much more slowly than is oxygen (Douglas, unpublished results). The experimental results presented in Table 1 and the times taken to obtain constant final readings in the Haldane apparatus indicate that this method determines the total oxygen plus CO, over the range of CO contents found in the blood of smokers, 0–2 vol. %.

It is evident that the CO content of the blood of smokers may be quite considerable (as shown also by other observers) and that the number of cigarettes smoked by any individual is not an indication of the CO level of the blood—a heavy smoker may have a low CO content while a light smoker may have a high CO content during the course of a day. Even in a non-smoker there may be some CO in the blood, probably absorbed from exhaust fumes of internal combustion engines or leaky gas fires.

These facts should be borne in mind whenever oxygen is determined in human blood, e.g. when the total blood haemoglobin is determined by measuring the oxygen capacity by the van Slyke method or when a haemoglobinometer is standardized by this method. In these cases, if the van Slyke apparatus is used, the CO capacity of the blood should be determined, or if the oxygen capacity is measured, the CO content of the blood should also be estimated and added on to the value obtained for the oxygen capacity. For these purposes, therefore, the Haldane apparatus is probably preferable.

If arterio-venous oxygen differences are to be determined, then either apparatus should give the same result. The van Slyke apparatus will determine the true oxygen content of both arterial and venous blood, whereas the Haldane apparatus measures the oxygen plus CO content of each sample. Since at any one time the CO content of the arterial and venous blood will be the same, the true arterio-venous oxygen difference will be measured.

When an oxygen dissociation curve is determined with blood which contains an appreciable amount of CO, then neither apparatus alone will give a true picture. For example, in a sample of blood with total oxygen or CO capacity of 20 ml./100 ml., and containing 9 ml. oxygen (combined with haemoglobin) and 1 ml. CO/100 ml., the haemoglobin will be 45 % saturated with oxygen and 5 % saturated with CO. By the van Slyke method the oxygen content will be 9 and the oxygen capacity 19 ml./100 ml., giving a percentage saturation of haemoglobin of 47·4. The Haldane apparatus will give an oxygen content of 10 ml./100 ml. and an oxygen capacity of 20 ml./100 ml. with a resultant percentage saturation of haemoglobin of 50. Thus if the oxygen dissociation curve of the blood of a smoker is required, or if any shift in his dissociation curve is being determined under different physiological conditions, the fact that the blood may contain up to 1·5 or more vol. % of CO must be appreciated, whichever apparatus is used.

The dissociation curves of Courtice & Douglas (1947) were obtained with blood of subjects who had a very low CO content (0·2 vol. % before equilibration in the tonometer) as the blood was always withdrawn at least 12 hr. after smoking. The errors due to CO, therefore, must have been very small, but this very small amount of CO may account for the fact that the Haldane apparatus gave, if anything, very slightly higher results on the average than the van Slyke.

SUMMARY

1. The Haldane method of blood-gas analysis measures the total active haemoglobin when blood containing up to 2 vol. % of CO is analysed. The figures obtained by this method for oxygen capacity agree with those for oxygen capacity by the van Slyke method plus the CO content.

2. The CO content of the blood of smokers may be quite considerable, up to 1·6 vol. % in these investigations, and varies with the individual's method of smoking rather than with the number of cigarettes smoked.

3. The blood of non-smokers in Oxford contains small amounts of CO, up to 0·2 vol. %.

4. The errors involved by the presence of CO when either the Haldane or van Slyke apparatus is used have been discussed.

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SOME EFFECTS OF NICOTINE-LIKE SUBSTANCES AND THEIR RELATION TO SENSORY NERVE ENDINGS

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Peripheral effects of substances acting like nicotine have already been demonstrated with certainty at two sites, the receptive area of the ganglion cell and the motor end-plate. Evidence suggesting that nicotine-like substances act at sensory nerve endings is presented in this paper. Sensory endings resemble the other sites at which nicotine is known to act in that they are places at which propagated impulses are initiated. The possibility of such nicotine-like action was suggested by Coon & Rothman's (1940) description of an axon reflex initiated by acetylcholine. They showed that intradermal injections of acetylcholine caused an erection of hairs which was not confined to the site of injection and was not abolished by atropine. It disappeared after degenerative section of the post-ganglionic sympathetic supply to the area examined.

Our experiments have shown that the close arterial injection of acetylcholine or nicotine into skin and mesentery causes a discharge of impulses in the sensory nerves, and evidence is given that this may be a direct effect upon the endings exposed to the drugs.

A preliminary note on the experiments described here has already been published (Gray, 1947*a*).

METHODS

The experiments have been carried out on dogs and cats anaesthetized with nembutal or chloralose and also on cats decerebrated under ether. Preparations of skin and mesentery were used.

Skin preparation. Either the skin over the external aspect of the lower part of the thigh or that over the internal aspect of the upper part of the thigh was used. The main cutaneous vessels of the region were dissected and a convenient small branch, supplying a circumscribed area, was chosen. The nerve associated with the smaller vessel was freed from connective tissue and divided for subsequent recording from its peripheral end. A fine hypodermic needle without bevel was then inserted, in a central direction, and tied into the main artery peripheral to the origin of the side branch. The main artery, central to the side branch, was cleaned to receive a bull-dog clamp, and all other branches between the cannula and the eventual site of the bull-dog clamp were double-tied and divided. The nerve alongside the branch artery was then placed on fine brush electrodes, which were moistened with Locke's solution and bound lightly, almost to the tips, with a fine silver wire. In some preparations the whole area of skin supplied by the small artery and nerve was dissected free, except for a pedicle consisting of the artery, vein and nerve (Fig. 1).

Before an injection the butt of the needle was washed out with the solution to be used and the syringe was fitted. The artery was then occluded with a bull-dog clamp, central to the branch, and the solution was injected rapidly. Except during an injection, the blood supply to the preparation was unobstructed.

One experiment was done on a cat after abdominal sympathectomy. Under ether anaesthesia, and with full asepsis, the abdomen was opened and the sympathetic chains on both sides from L3 to L7 were removed; 4 weeks elapsed before the final experiment was done.

Mesentery preparation. A loop of mesentery was laid over a silver plate and a mesenteric nerve was dissected just peripheral to the lymphoid tissue at the mesenteric root. The nerve was cut centrally and placed on the electrode (Fig. 2). The artery lying alongside it, or a branch, was then cannulated. In the latter case the artery was prepared for occlusion central to the junction. In some preparations, all visible arterial branches running between the mesenteric arch and the intestine were ligated. The technique of injection was that described above.

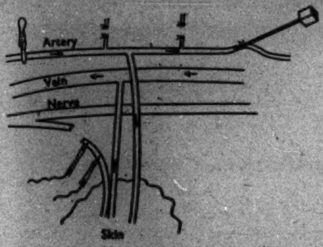

Fig. 1.

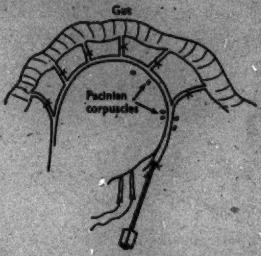

Fig. 2.

Fig. 1. Diagram to show the arrangement of the cannula, bull-dog clamp and electrodes in the skin preparation. The arrows mark the direction of the normal blood flow.

Fig. 2. Diagram to show an arrangement of the arterial cannula and electrodes in a mesentery preparation.

Single Pacinian corpuscle preparations. The preparation was that described previously (Gray, 1947*b*), with the addition of arterial cannulation as described under the mesentery preparation. Injections were made into the mesenteric vessel while recording from the corpuscle.

In all experiments the animal was kept in a metal box, the atmosphere in which was kept moist and at a temperature around 35° C.

Recording. A resistance-capacity coupled amplifier connected to a Matthews or cathode ray oscillograph was used for recording on moving paper. In some experiments, photographs were taken of sweeps on a cathode ray oscillograph, the sweeps being synchronized with the time marker on the continuous paper record. This procedure allowed high velocity samples to be taken at regular intervals during the slow-moving record. A description of recording technique has already been published (Gray, 1947*b*).

Solutions. Stock solutions of nicotine tartrate 1 mg./ml. and 10 mg./ml. were made up in 0·9% sodium chloride solution, made just acid to Congo Red with hydrochloric acid and kept at −4° C. Before using for arterial injection the stock solution was diluted with Locke's solution.

In some experiments, fresh solutions of crystalline acetylcholine chloride, made up in 0·9% sodium chloride and Locke's solution, were used, but a stock solution of 10 mg. of acetylcholine in 1 ml. of 5% sodium dihydrogen phosphate solution was kept in the cold store and after dilution with Locke's solution was used for arterial injection in most experiments. In the latter case a corresponding dilution of 5% phosphate was injected as a control.

The other drugs for arterial injection were kept as 0·1 or 1% solutions in acidified 0·9% sodium chloride solution, and diluted with Locke's solution before use.

RESULTS

Skin and mesentery preparations

Touch. In all preparations, touching the skin or mesentery with an insulated bristle gave good responses in the nerve, although the fibres responding did not include all those excitable by other means. Spontaneous discharge from a few fibres was common, and in the mesentery it occasionally followed a cardiac rhythm, a phenomenon described by Gammons & Bronk (1935). Crushing or tying the nerve between the preparation and the electrodes eliminated all responses.

Pressure response. In most preparations, injection of any liquid set up a brief discharge during the actual injection. These responses vary very much from preparation to preparation, but during a single experiment remain constant in form (Fig. 3*b*). It is certain that these are responses to intravascular pressure change (cf. Gammons & Bronk, 1935). They can be produced by Locke's solution and even by blood. In one preparation, which showed a particularly good response, blood was withdrawn from the carotid artery into a warmed syringe and immediately reinjected into the cannula. This set up the characteristic pressure response.

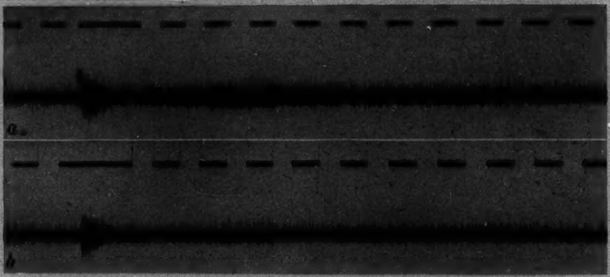

Fig. 3. Dog, 9·2 kg. Nembutal. Records from a cutaneous nerve. Time, 1 cyc. = 0·5 sec. (*a*) 0·2 ml. of 1/10,000 nicotine tartrate was injected during the signal (continuous line in time record). (*b*) Control injection.

Usually the discharge evoked by an injected drug overlaps and largely obscures the pressure response, but occasionally, particularly when there was an unusually large volume of artery between the cannula and the piece of skin, the two responses were discrete (cf. Fig. 3*a*).

Excitatory effect of nicotine. Nicotine sets up a centripetal discharge of action potentials in both cutaneous and mesenteric nerves. Fig. 3*a* shows the effect of the injection, into the skin preparation of a chloralosed dog, of 0·2 ml. of 1/10,000 nicotine tartrate (i.e. 1/31,000 nicotine base). A pressure response occurred during the injection; after the pressure response came a pause of 0·5 sec. duration and this was followed by a further discharge of action potentials. The duration of the activity was somewhat over 8 sec. The latent period and duration of the discharge varied very much with different preparations and were presumably dependent on such variables as speed of injection and volume of dead space, as well as on the condition of the vascular bed of the preparation. Fig. 3*b* shows a control injection done immediately after Fig. 3*a*.

Fig. 4*a* shows a record of a similar injection into a cat's mesentery preparation, the intestine being included. The time marks represent 0·5 sec. and at every up-stroke a single sweep on a cathode ray tube was photographed. Two of these are shown in Fig. 4*b* and *c*, and give a more detailed picture of the discharge. They suggest that the bulk of the activity is in the smaller fibres, and Fig. 4*c* is similar in appearance to the records obtained by Gernandt & Zotterman (1946) on pinching the intestine.

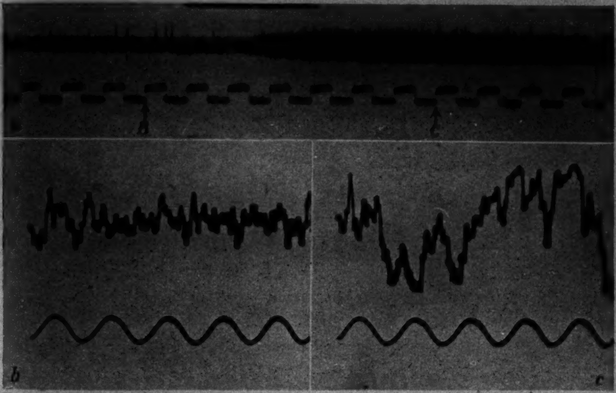

Fig. 4. Cat, 2·5 kg. Chloralose. Atropine 2 mg. Records from a mesenteric nerve. (*a*) 0·2 ml. of 1/10,000 nicotine tartrate was injected during the signal (the dark line above the record). The large spikes before the signal are artefacts. Time, 1 cyc. =0·5 sec. At every upstroke of the time signal, a single sweep on the oscillograph was photographed. (*b*) The single sweep photographed at the upstroke marked *B* in record (*a*). Time 300 cyc./sec. (*c*) The single sweep photographed at the upstroke marked *C* in record (*a*). Time 300 cyc./sec.

Excitatory effects of acetylcholine. Acetylcholine chloride, when injected in the same way and also in a concentration of 1/10,000, has an effect indistinguishable from that of nicotine tartrate. Fig. 5*a* is an example of a record taken during the injection of 1/10,000 acetylcholine into a mesentery preparation in a cat. Fig. 5*b* and *c* are photographs of sweeps synchronized with the time mark of Fig. 5*a* and show the discharge in more detail.

With acetylcholine injections, it was possible that the sensory discharge observed might be secondary to the stimulation of smooth muscle by the muscarine-like action of the drug. This action, however, can be blocked by atropine (Dale, 1914).

Atropine. This does not abolish the responses described. The dog (9·2 kg.) used for the experiment illustrated in Fig. 3 had received 1 mg. atropine sulphate, subcutaneously, 5 hr. before, while the cat (2·5 kg.) of the experiment shown in Fig. 4, had received 2 mg. intravenously 1 hr. previously. Fig. 6*a*, discussed in more detail below, was also recorded after atropine had been administered (1 mg. for a 2·7 kg. cat).

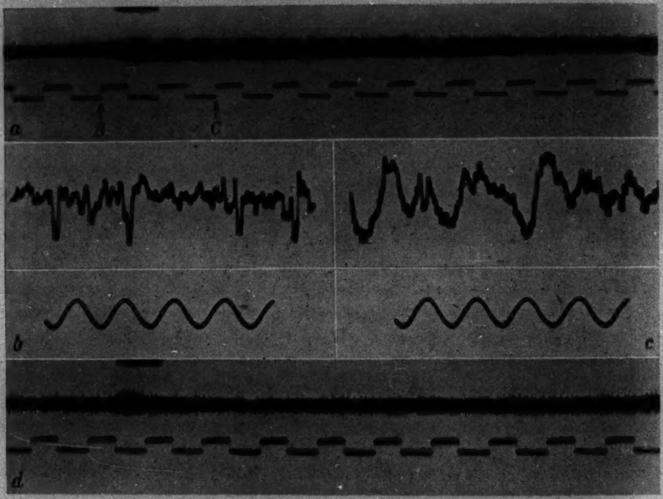

Fig. 5. Cat, 2·4 kg. Chloralose. Records from a mesenteric nerve. (*a*) 0·5 ml. of 1/10,000 acetylcholine chloride was injected during the signal (the dark line above the record). Time, 1 cyc. = 0·67 sec. At every upstroke of the time signal, a single sweep on the oscillograph was photographed. (*b*) The single sweep photographed at the upstroke marked *B* in record (*a*). This shows a few fast potentials from the beginning of the 'pressure response'. Time, 300 cyc./sec. (*c*) The single sweep photographed at the upstroke marked *C* in record (*a*). Time, 300 cyc./sec. (*d*) As record (*a*), but 0·5 ml. of 1/1000 acetyl-β-methyl choline was injected.

In one experiment on a mesentery preparation a late response occurred some seconds after the injection, with a distinct interval between it and the ordinary response. The 'secondary' response disappeared after atropine and appeared to coincide with the intestinal movement which was set up.

This evidence suggested that the effect of acetylcholine was due to a direct action on the sensory neurones and not to secondary excitation of sensory fibres as a result of the stimulation of the intestine or other smooth muscle. As a further control, the effects of acetyl-β-methyl choline and histamine were examined, since both cause contraction of smooth muscle, but are without nicotine-like effects (Simonarts, 1932).

Acetyl-β-methyl choline. Neither 1/10,000 nor 1/1000 acetyl-β-methyl choline produces the response. Fig. 5*d* is a record taken while 0·5 ml. of 1/1000 acetyl-β-methyl choline was injected into the same preparation as that from which Fig. 5*a–c* was taken. A pressure response only is seen.

Histamine. Histamine, like acetyl-β-methyl choline, was without effect, solutions of 1/1000 and 1/10,000 producing no response.

Carbaminoyl choline. 1/1000 carbaminoyl choline produced a response indistinguishable from that produced by 1/10,000 acetylcholine or nicotine. 1/10,000 carbaminoyl choline gave no response.

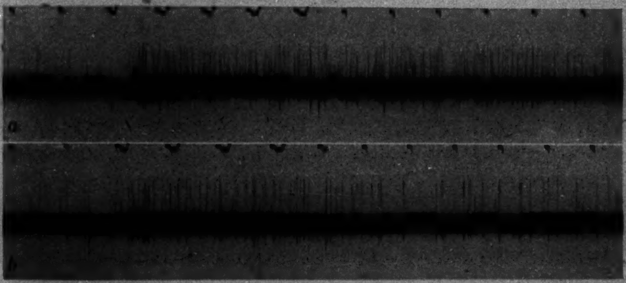

Fig. 6. Sympathectomized cat, 2·4 kg. Nembutal. Atropine 1 mg. Records from a cutaneous nerve. Time, 0·5 sec. (*a*) 0·2 ml. of 1/10,000 acetylcholine chloride was injected during the signal (the change in time-mark shape). (*b*) Same preparation. Control injection.

It seems certain, therefore, that the response is not due to smooth muscle contraction, either set up by the muscarine-like action of the drugs or by post-ganglionic stimulation at parasympathetic synapses in the mesentery preparation. Such synapses are not known to occur in the skin of the limbs and so the skin preparation may be regarded as a control of this point. Skeletal muscle is absent from both preparations, with the possible exception that, in some of the cats, a few fibres of the cutaneous maximus muscle may have extended into the skin preparation. It is unlikely that this occurred in any of the dogs. The only other motor effect which could be involved is the pilomotor reflex described by Coon & Rothman (1940) and, according to them, this is lost after degeneration of the post-ganglionic sympathetic supply to the part.

Sympathectomy. Degeneration of the post-ganglionic sympathetic did not affect the response from the skin. Fig. 6*a* shows the response, to an injection of 0·2 ml. of 1/10,000 acetylcholine, of a skin preparation in a cat which had

had both lumbar sympathetic chains removed a month before. The preparation used was supplied by branches of the saphenous nerve and artery, and it was established at post-mortem that all ganglia between L3 and L7 had been removed. Fig. 6*b* shows a control injection into the same preparation.

Potassium. Potassium chloride injected into both these preparations has a very strong stimulating effect. Brown & MacIntosh (1939) showed that K^+ can stimulate nerve trunks, and this technique can, therefore, give no information about the effect on nerve endings. Its stimulating action has, however, proved very useful in checking the viability of preparations. Either Locke's solution with all Na^+ replaced by K^+, or as suggested by Shanes (1946), Locke's solution plus 1·2% KCl was used. No difference in the results produced by the two solutions was noticed. In some cases these solutions produced an inhibition, in which case the response to touch was lost.

Inhibition. Intravenous injections of paralysing doses of nicotine tartrate (30 mg.) into cats whose mesentery preparations were giving good responses to injections of 1/10,000 nicotine or acetylcholine, abolished these responses. K^+ produced a big response after nicotine paralysis. Just before natural respiration returned, 30 min. after the intravenous nicotine, the responses to nicotine and acetylcholine were beginning to return. An hour after the injection the responses were back to their pre-injection level.

Abolition of nicotine and acetylcholine responses has also been produced by the injection of 1/1000 acetylcholine into the arterial cannula of the skin preparation of a dog and the mesentery preparation of a cat after these animals had received intravenous eserine. In these cases the preparations remained active to K^+.

The response to pressure on the skin was not obviously affected by nicotine in paralysing doses, or by doses of acetylcholine which abolished the effects of stimulating doses of nicotine or acetylcholine. The inhibition produced by nicotine and acetylcholine is thus different from that produced by potassium. The significance of these results is discussed below.

Eserine. No response has ever been observed to the injection of eserine into the arterial cannula. On one occasion eserinization of the cat increased the duration of the response to an injection of 0·2 ml. of 1/10,000 acetylcholine by many times. The animal had previously received 2 mg. of atropine intravenously. In other preparations eserinization of the whole animal produced no effect on the acetylcholine response.

Fibre size

There is a certain amount of evidence to indicate the size of the nerve fibres stimulated. Fig. 3 shows that the smaller fibres are involved, and, particularly early in the response, the larger fibres are probably involved also. It is, however, difficult, in the type of record illustrated, to distinguish faster fibres from the synchronous firing of slow ones. Fig. 5, in which the action potentials remain distinct, shows clearly that the larger fibres as well as some of medium size are involved. Figs. 4*c* and 6*a* show that many small fibres are responding. These figures are very similar to those published by Gernandt & Zotterman (1946), who suggest that the waves are made up of potentials from the slower fibres of the δ group and possibly also *C* fibres. Evidence for larger fibre responses in the mesentery has been hard to find, although certain records of superimposed sweeps, taken with high sweep velocities, do suggest that a large fibre response can be present. In many other experiments, however, it clearly was not present, while, in one, a similar recording technique suggests an inhibition of the larger fibre responses.

Single Pacinian corpuscle preparations

Three preparations of single corpuscles were made and injections into these failed to produce any response of the corpuscle or the axon to any substance including potassium chloride. The preparations were in other respects satisfactory; mechanical stimuli produced corpuscular action potentials; stimulation of the mesenteric nerve produced antidromic action potentials at the corpuscle; the injected solutions could be seen in vessels close to the corpuscle, and recording from the mesenteric nerve during injections showed that other endings were being stimulated. Potassium chloride, however, failed to cause a discharge from the corpuscle or its axon, and this failure of KCl to excite the preparation suggests that none of the injected solution was reaching either the corpuscle or its nerve. As Brown & MacIntosh (1939) have shown, KCl in suitable concentrations can cause discharges in pre- and post-ganglionic sympathetic fibres and even in the fibres of a large motor nerve; and it was to be expected that, even if the corpuscle itself was inaccessible or unexcitable, some solution would reach and stimulate its afferent axon. In the conditions of our experiments such excitation of the axon would have been easily detected, since the corpuscle displayed large potential spikes in response to stimulation of the appropriate mesenteric nerve with single shocks. The artery supplying the corpuscle is a fine vessel which arises from a much larger mesenteric artery and runs through the mesentery approximately at right angles to its artery of origin. Dissection and ligation of branches further restricts the blood flow, and it seems possible that a solution injected into the main vessel may not reach the nerve and corpuscle in a stimulating concentration. That this failure to obtain large-fibre responses to potassium is not confined to single Pacinian corpuscle preparations is shown by experiments in which all visible arteries running between the mesentery and intestine were ligated. This procedure reduced the small-fibre response considerably so that the wave peaks were small compared with the large-fibre action potentials. In spite of good responses to mechanical stimuli, including pressure responses from these fibres, no responses were observed when potassium was injected. Small-fibre responses, though reduced, were present after injection of both potassium and acetylcholine.

Acetylcholine content of Pacinian corpuscles

Pacinian corpuscles were dissected from the mesenteries of cats which had received 1 mg./kg. of eserine. They were weighed and extracted with 10% trichloracetic acid (Brown & Feldberg, 1936). After filtering and washing with ether, the extract was tested on the cat's blood pressure and on leech muscle. The corpuscles contained no more active substance than the mesenteric fat.

DISCUSSION

Our experiments show that a centripetal discharge of action potentials occurs when nicotine or acetylcholine is injected into mesentery or skin. It appeared regularly in the skin preparation and this would appear to set beyond question its derivation from sensory fibres, since the preparations we used were innervated by the saphenous nerve, the fibres of which have been shown by Heinbecker, O'Leary & Bishop (1933) to be almost exclusively sensory, their motor component being confined to a few small non-medullated fibres arising probably from the post-ganglionic sympathetic system. It is suggested that there are three possible causes for such a response: (1) that the axons are stimulated; (2) that it is secondary to motor effects; (3) that it is set up at sensory endings.

As regards the first possibility, Lorente de Nó (1944) failed to stimulate isolated frog's nerve even with isotonic acetylcholine. Hodgkin (1947), in a few preliminary experiments, showed that 1% acetylcholine chloride did not appreciably alter the membrane resistance of the isolated crab axon, and Arvanitaki & Chalazonitis (1947) failed to excite action potentials in the giant fibre of sepia with acetylcholine. In the intact animal, Brown & MacIntosh (1939) failed to set up action potentials in nerves by intra-arterial injection of acetylcholine, although they consistently succeeded with K^+. In the face of this evidence it is impossible to regard the effect as a direct action on the whole length of the axons.

With regard to the second possibility, there is no reason to suppose that the effects which we have observed were due to sensory impulses set up by the excitation of other structures in our preparations. They persist after doses of atropine sufficient to annul the stimulant effect of acetylcholine on smooth muscle, and they are not evoked by substances like acetyl-β-methyl choline and histamine which excite smooth muscle, but have no nicotine-like properties. The pilomotor reflex of Coon & Rothman (1940) does not appear to participate in the responses from skin, since they persist after sympathectomy, which abolishes the reflex. The responses which we have obtained have properties which recall the reactions of peripheral ganglionic synapses and of skeletal muscle to nicotine-like substances, and it is conceivable that they might be due to secondary effects resulting from stimulation of such tissues in our preparations. Against this must be put the fact that neither preparation contains skeletal muscle, and ganglion cells have never been described in the skin of the thigh, though they do occur in the intestine.

After the exclusion of other possible causes, the only remaining explanation is that the effect is on the sensory endings.

Our failure to elicit responses from single Pacinian corpuscles does not, in our opinion, weigh very heavily against such a conclusion, since the absence of an excitatory effect of KCl under the same circumstances indicates clearly that there is some fault in the technique used for these isolated nerve endings.

A more serious objection is that a paralysing dose of nicotine does not affect sensory responses from the skin, although it abolishes the responses to nicotine or acetylcholine. It follows that if nicotine inhibition consists of complete depolarization, that is, a complete obstruction to an action potential, then the site of its action can only be outside the neurone or at the tip of the axon. In the latter case normal mechanical stimuli must act on areas other than those sensitive to nicotine. That these substances act outside the neurone and stimulate the sensory endings indirectly is an explanation that cannot be reconciled with the experimental evidence. Another explanation which fits all the experimental data is that nicotine does not paralyse by a complete blocking of normal conduction, but by a mechanism which is specific against the stimulating properties of itself and similar substances. These difficulties may be overcome if it is possible to improve the technique for handling isolated sensory endings.

We have no reason to suppose that the discharge is confined to any specific type of nerve fibre: in the skin certainly, all fibre types, large and small, are involved. For the mesentery the evidence is inconclusive, and we are as yet unable to state whether or not large-fibre responses are produced by the injection. According to Gernandt & Zotterman (1946) there are about 300 large fibres and 10,000 small myelinated fibres, apart from a large number of non-myelinated fibres, in the splanchnic nerve, and it is probable that the semi-synchronized firing of small fibres obscures the discharge of the large fibres. The experiments on isolated Pacinian corpuscles failed to elucidate these points, but did serve to show that hydro-dynamic effects may, by themselves, introduce a type of differentiation of response which has nothing to do with the sensitivity of the component cells of a tissue to substances reaching it through its blood stream.

In these experiments the concentrations used have been somewhat stronger than those found adequate to stimulate motor-end plates or ganglion cells, but the essential point is that these concentrations are many times weaker than those which failed to stimulate axons along their length. Exact comparisons of concentrations are impossible as it is the concentration at the site of action which is important, and this must depend on the velocity and pressure of injection, the amount of vascular dead-space and the volume of tissue fluid. The last point is certainly variable as some degree of oedema was likely to occur.

The significance of our observations appears to be that sensory endings, or a part of such endings, have properties which are distinct from those of axons, but very similar to the receptive parts of ganglion cells and motor end-plates. The work on chemical transmission suggests that this specialization is associated with the initiation of propagated impulses. Whether the distinction between the sensory ending and its axon is one of accessibility or of membrane property is a question that remains to be answered.

Our experiments do not suggest that acetylcholine is involved in the normal function of sensory endings, and do, in fact, provide reasonably conclusive evidence against such participation. The fact that nicotine inhibition does not interfere with normal sensory stimulation is the most important evidence, but is supported by the absence of acetylcholine from the Pacinian corpuscle.

By drawing on evidence obtained with different types of nerve, it is possible to speculate on some of the properties of a neurone associated with sensitivity to nicotine-like substances. The effects of nicotine-like substances on the axon have already been considered. Brown & MacIntosh (1939) injected acetylcholine into a sensory ganglion, that of the vagus, and obtained no response in the nerve. They also injected acetylcholine into the superior cervical ganglion and obtained no response in the pre-ganglionic fibres. It appears then that there is a definite orientation of a neurone apart from its connexions. The receiving end, e.g. the central end of a post-ganglionic visceral motor fibre, or the peripheral end of a sensory nerve, is sensitive to nicotine-like substances. The transmitting end, e.g. the peripheral end of a pre-ganglionic visceral motor fibre, is not sensitive to nicotine-like substances, but can, in certain circumstances, liberate acetylcholine. The intermediate part of the neurone, including the cell body, is insensitive. In the case of the post-ganglionic visceral motor neurone, the cell body is at the receiving end of the neurone and happens to be associated with the nicotine sensitive area.

SUMMARY

1. Arterial injection of nicotine and acetylcholine into the skin or mesentery of cats and dogs, causes a centripetal discharge of impulses in the nerves supplying the injected area.

2. The response is not abolished by atropine and is not elicited by acetyl-β-methyl choline or histamine.

3. Large doses of nicotine and large doses of acetylcholine, after eserine, abolish the sensitivity of the preparation to subsequent doses, but not to mechanical stimuli.

4. The impulses probably arise through the direct chemical stimulation of some part of the terminations of the sensory nerves, although we have failed to obtain them from single Pacinian corpuscles.

Our thanks are due to Dr F. C. MacIntosh for his assistance with the assay of acetylcholine in Pacinian corpuscles and to Mr D. G. A. Thomas, of T. R. E., Malvern, for considerable help in the design of electronic equipment.

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SOME PROPERTIES OF AMARIN, WITH SPECIAL REFERENCE TO ITS USE IN CONJUNCTION WITH ADRENALINE FOR THE PRODUCTION OF IDIO-VENTRICULAR RHYTHMS

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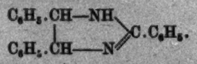
Amarin is the name by which the synthetic base 2:4:5-triphenyldihydroiminazole (I) has long been known, probably on account of its bitter taste. The properties of amarin have so far been investigated almost exclusively from a chemical standpoint; only two references to its pharmacological effects appear in the literature. Bülow (1894) reported its poisonous action. His results were confirmed by Auvermann (1919) who showed that in the rabbit a lethal dose (20 mg. or less) produced a rapid fall of blood pressure. Amarin was found to cause relaxation of strips of rabbit's intestine.

(I) 

$$\begin{array}{l} C_6H_5.CH\text{—}NH \\ \quad\;\;| \\ C_6H_5.CH\text{——}N \end{array} \!\!>\! C.C_6H_5.$$

The property with which the present report is mainly concerned is inconspicuous, if present at all, in lower amidine derivatives described elsewhere (Fastier & Smirk, 1943, 1947). This property, which had been noted previously by one of us (F.H.S.) in the course of an examination of the circulatory effects of amarin, is a capacity to increase the liability of the heart to disturbances of rhythm such as heart block, premature ventricular systoles, ventricular tachycardia, ventricular flutter and ventricular fibrillation. It is thought to provide a useful tool for the investigation of these arrhythmias. The experiments now to be described centre around the production and nature of ventricular flutter and fibrillation, but they have also furnished results which seem to bear upon the refractory period of cardiac muscle.

METHODS

Dogs and cats were anaesthetized with sodium barbitone. Cannulae were placed in a femoral vein, to facilitate intravenous injection, and in a carotid artery for measurements of blood pressure with a mercury manometer. Electrocardiograms were usually either observed directly on a Cossor-Robertson oscillograph or recorded photographically from it. Stainless steel electrodes were used with the usual limb leads. A record of the respiratory excursions was obtained by connecting a stethograph, placed around the animal's chest, to a tambour.

In a number of experiments, the movements of the heart were observed by opening the thorax medially and removing the pericardium; artificial respiration with positive pressure was maintained meanwhile with an 'Ideal' respiration pump. At the same time an electrogram from a direct lead hooked on to the ventricular surface was recorded on a double beam oscillograph, which was constructed so that each beam was fed from a battery-operated R.C. coupled amplifier consisting of double triode differential input and four stages of 6SJ7 pentodes coupled through 4 μF. condensers and 5 MΩ. grid leaks; 0·05 μF. plate by-pass condensers producing a bandwidth from about 1/10th cycle to 100 cycles. This instrument permitted two records to be obtained simultaneously. In some experiments, an ordinary lead 2 electrocardiogram was taken in addition to the electrogram; in others, a second electrogram was picked up with a movable nickel-plated electrode with an insulated handle which could be slid over the ventricular surface without breaking electrical contact with it during episodes of ventricular flutter. The nature of the 'flutter' movements was further studied by analysing cinematograph records which had been filmed at high speed (4 × normal) in the course of several of these experiments. Mechanograms were taken with a myocardiograph working on the same principle as that described by Cushny (1911) except that the moving arms pressed on tambours, using air transmission through thick rubber tubes to recording tambours to obtain the tracing.

Amarin hydrochloride (m.p. 298° C. uncorr.) was obtained by heating hydrobenzamide for 3 hr. at 120–130° C. and treating an alcoholic solution of the product with concentrated hydrochloric acid (Bertagnini, 1854). Because of their insolubility in aqueous solution, neither the salt nor the free base (m.p. 128° C.) proved easy to administer. The base was injected in 10% alcoholic solution. Like the hydrochloride, it tended to flocculate when washed into the circulation with salt solution.

RESULTS

General effects of amarin in animals

Pulmonary ventilation was increased by amarin; sometimes spontaneous breathing was restored in deeply anaesthetized dogs and cats. Central stimulation was also indicated by such effects as the re-establishment of the conjunctival reflex and reappearance of muscular movements. Generalized clonic convulsions were induced in anaesthetized and unanaesthetized rats and mice by subcutaneous and intraperitoneal injections of amarin. The substance has an appreciable analeptic action and this will be described more fully in a separate communication. A small pressor response, which was sometimes quite prolonged, was the effect observed most often in dogs when the amount of amarin injected initially was small (1–3 mg./kg.). Larger doses were generally depressor. Injections of the order of 10 mg./kg. rapidly produced circulatory collapse with fall of blood pressure, cardiac dilatation and arrest of the heart beat.

It was evident from direct inspection that the administration of amarin leads to great slowing of the heart. Experiments carried out after vagal section showed that amarin slows the auricles in dogs and cats by a non-vagal mechanism.

In a series of about thirty experiments on anaesthetized dogs it was found that substantial doses of amarin (4–8 mg./kg.) nearly always enhanced considerably the pressor response to small doses (1–5 μg./kg.) of adrenaline. Often a comparable rise of blood pressure could be secured with 1/5th or, exceptionally, 1/20th of the amount of adrenaline used before the amarin. The response to a larger dose of adrenaline was modified in a totally different manner; as will be seen in the next section, its effect was to precipitate ventricular flutter, which was followed, as is normally the case, by ventricular fibrillation.

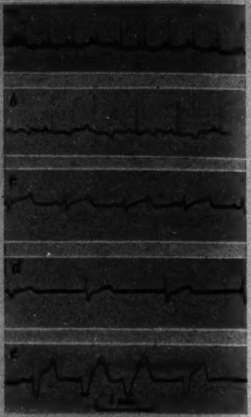


Fig. 1. Lead 2 electrocardiograms. Dog: 13 kg., 3·8 g. sodium barbitone. Vagi intact. (*a*) Control electrocardiogram; (*b*) after 70 mg. of amarin; (*c*) after 180 mg.; (*d*) after 200 mg.; (*e*) later stage after 200 mg. in all of amarin. On all electrocardiograms and electrograms, 2 cm.=1 sec. Note the striking increases in the *PR* interval and in the duration of the *QRST* complex which accompany the great slowing in the rate of beating (*a*–*c*). A slow nodal rhythm appears at (*d*), with the ventricular complex hardly altered from (*c*) except that a low *P* wave occurs shortly after the *S* wave. The next change in rhythm (*e*) occurred without further injections of amarin. The ventricular complexes suggest that the point of origin of the beat now lies in or peripheral to one of the branch bundles.

The effect of amarin alone (i.e. without adrenaline) on the electrocardiogram is to prolong the duration of the ventricular complex, often to two or three times the normal (Figs. 1*a*, *d* and 6*a*, *b*), and this contributes largely to a decrease in the heart-rate (Fig. 1*b*). Electrocardiograms also show prolongation of the *PR* interval (Fig. 1*b*, *c*). With the larger doses, the rhythm of the heart may take its origin from the auriculo-ventricular node (Fig. 1*d*) or from a ventricular focus (Fig. 1*e*). In one and the same animal qualitatively different effects may be observed as more amarin is given. Such abnormalities as partial or complete heart block, branch bundle block, ventricles driving the auricles, the two auricles beating independently, elevation or depression (Fig. 1*b*) of the *ST* segment, great enlargement of the *T* wave or reversal (Fig. 1*c*) of its direction and cardiac alternation, both mechanical and electrocardiographic, have all been observed on occasion.

The production of ventricular flutter and ventricular fibrillation by amarin and adrenaline

Administration of amarin in doses of the order of 5 mg./kg. leaves the circulation in apparently good condition, although the heart is slow. After the amarin, however, moderate doses of adrenaline cause the heart to beat with chaotic irregularity and the initial blood pressure rise is interrupted by a precipitous fall to zero. This is due to the sudden onset of ventricular flutter. The phenomenon was readily obtained. In a series of upwards of 80 dogs, less than half a dozen proved refractory, and the dose of adrenaline needed to produce ventricular flutter was small in comparison with that usually tolerated. Thus with the first 19 dogs the 'lethal' doses of adrenaline were 2·5, 3·1, 0·9, 2·0, 14·0, 2·6, 3·6, 1·9, 2·4, 1·8, 1·7, 1·0, 0·9, 4·7, 1·9, 1·1 and 3·7 μg./kg. respectively, the two remaining animals being refractory. Ventricular flutter was also obtained in several dozen cats.

Phenomena preceding the onset of ventricular flutter or fibrillation. After the injection of adrenaline, but before the development of ventricular flutter, there almost always appeared in the electrocardiogram the numerous polymorphic ectopic ventricular complexes (Fig. 2) described by other workers as the state of 'chaotic rhythm'. This stage of 'chaotic rhythm' was usually succeeded by the well-known undulatory curve (Fig. 2*b*) that characterizes the electrocardiogram of ventricular flutter. In other cases 'chaotic rhythm' was followed by a return to normal rhythm.

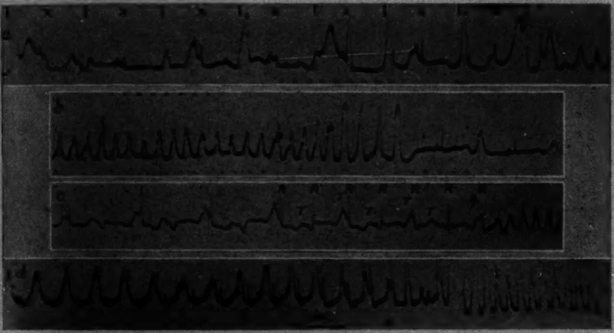


Fig. 2. Lead 2 electrocardiograms showing onset of ventricular flutter; appearance of *R* waves on *T* waves. (*a*, *b*) Cat: 3·3 kg., 1·5 g. sodium barbitone. Vagi intact. The rhythm preceding flutter is less chaotic than it would appear at first sight. Two recurring cycles can be made out. The *R*-like waves marked x are equally spaced, and although the complexes marked I do not occur quite so regularly, they too are approximately equidistant. In fact, until a few cycles before the onset of flutter, all of the irregular waves fall into one of two systems. The *R*-like waves marked • would appear to mark the genesis of the new rhythm. The second of these actually lies on the *ascent* of a *T* wave. (*c*) Cat: 3·5 kg., 1·2 g. sodium barbitone. Vagi intact. Ventricular flutter induced with amarin (40 mg.) followed by adrenaline (5 μg.). Before the undulatory curve of flutter appears, *R* waves are to be seen on the descent of the antecedent *T* waves in alternate complexes. The height of the latter has been increased considerably by the amarin. (*d*) Dog: 21 kg., 6 g. sodium barbitone. Vagi out. Ventricular flutter induced with amarin (60 mg.) followed by adrenaline (40 μg.). First the *P* wave and then the *R* wave climb up the greatly hypertrophied *T* wave until, at the point x, the *R* wave is about two-thirds of the way up, whereupon flutter supervenes.

A curious phenomenon was noted before the inception of ventricular flutter in many experiments. This was the appearance of *R* waves on the descent of the antecedent *T* waves (Fig. 2*a*, *c*, *d*). Occasionally, as the heart accelerated in response to the adrenaline, the *PQRS* deflexions were seen increasingly higher up the descent of the antecedent *T* wave until, as the *R* wave approached the peak of the *T* wave, the heart's rhythm suddenly changed over to flutter (Fig. 2*d*).

The possibility that a *T* wave may be interrupted by the succeeding *R* wave (Fig. 2) challenges detailed explanation, as the interruption must be taken to mean that a new wave of excitation has arisen in the ventricles before the previous wave has subsided.

While numerous clear-cut examples of the phenomenon have been obtained in experiments with amarin, it has not proved easy to estimate how often the onset of ventricular flutter was definitely preceded by the appearance of *R* waves on *T* waves, owing to the difficulty of identifying the different electrocardiographic deflexions during the period of chaotic irregularity. However, we think it can be said that at this stage the phenomenon occurred more often than not.

Because of the more localized picture afforded by a lead directly attached to the heart, an electrogram was recorded at a time when the electrocardiogram would be expected to reveal the presence of *R* waves on *T* waves. As a rule, no corresponding phenomenon was provided by the electrogram. Exceptionally, however, an electrogram was obtained in which a few at least of the cycles seem to be interrupted before their completion in much the same manner as some of the *T* waves of the simultaneously recorded electrocardiogram are interrupted by the succeeding *R* waves. Further work on this is in progress.

Ventricular flutter. This condition was seen both in the cat and dog and was normally followed by the disorganized rhythm of ventricular fibrillation. Ventricular flutter is associated with characteristic regular undulatory waves on the electrocardiogram (Figs. 2, 5) and its onset is signalled by a precipitous fall in blood pressure.

It was observed, in agreement with other authors, that the rhythmical movements exhibited by the ventricles at this stage—that is, immediately following the premonitory chaotic irregularity—are of considerable amplitude, quite unlike the disorganized movements usually of smaller amplitude, which are customarily described as 'ventricular fibrillation' (Fig. 5*c*). A hint as to the nature and origin of these 'flutter' movements is given by the appearance of the heart (under amarin) when a premature ventricular systole occurs. The heart under amarin differs from the normal heart in the slow speed with which a premature contraction extends over the ventricular surface, the appearance resembling the spread of a peristaltic wave. This comparatively slow spread of the mechanical disturbance over the heart's surface facilitates direct observation also when ventricular flutter develops. It enabled us to see quite distinctly, and confirm with the aid of a slow-motion film, that in ventricular flutter a series of peristalsis-like waves succeed each other over approximately the same course on the ventricular surface, two or three such waves being visible simultaneously on whatever aspect of the ventricular surface was inspected. No single point could be discerned from which these waves appeared to take their origin; the continuous rhythmical activity of the heart gave the impression rather that the waves were tracing out 'circus' movements. It would seem that at this stage the relationship between the rate of conduction of the waves of excitation and the refractory period of the heart muscle had become such that, before any one wave of contraction could completely traverse the anterior surface of the heart, the fibres over which the wave first passed had ceased to be refractory, and could contract in response to the succeeding wave of excitation.

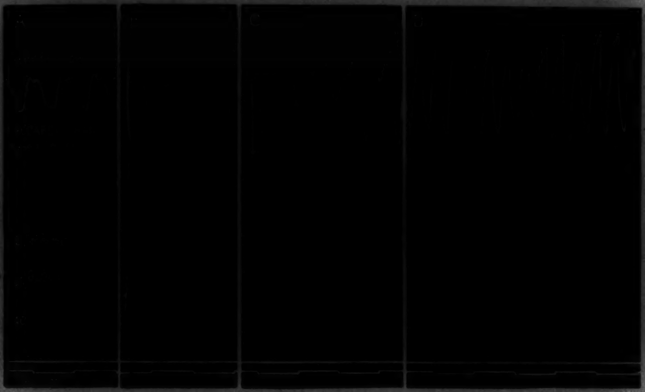


Fig. 3. The upper tracings were obtained with a double myocardiograph, the fixed central arm of which was placed upon the middle of the left ventricle; the movable arms were placed upon the apex and upon the base of the left ventricle respectively. Each myocardiogram records the distance between fixed points on the ventricular surface. (A), control, anaesthetized dog. (B), after amarin. Note the slower, more deliberate beat. (C), onset of ventricular flutter. The blood pressure rises at first in response to the injection of adrenaline but falls rapidly when flutter supervenes. (D), ventricular flutter. Note the characteristic undulatory curves of the myocardiograms. Meanwhile the blood pressure has declined practically to zero.

Myographic records from two selected points on the heart's surface showed that, during flutter, changes of considerable amplitude may be recorded which, however, differ from the records obtained when the heart is beating normally. Under normal conditions contraction is a rapid process, and relaxation takes place comparatively slowly. In flutter, contraction and relaxation take place at approximately the same speed and the mechanogram has the appearance of a sine curve, the individual cycles representing the passage of peristalsis-like waves over the part of the heart connected with the recording device (Fig. 3).

Proof of the visual impression that the waves of contraction which were seen to sweep across the portion of the ventricular surface under observation are parts of two or more cycles in progress simultaneously was sought in two ways. First, high-speed cinematographic records of ventricular flutter after amarin were projected at a normal speed, the more important sections being run over repeatedly. These films revealed the presence of at least two waves of contraction following the same course for the portion of the ventricular surface under observation but, naturally, involving different parts at one and the same time. By examining consecutive frames, the distance between successive wave fronts could be approximately measured in favourable cases. Secondly, electrograms were recorded simultaneously from two separate points on the ventricular surface by means of the double-beam oscillograph. In control electrograms (Fig. 4*a*, *b*) it can be seen that the wave of excitation invades the left ventricle as a whole since the pairs of traces are in phase not only when the two heart electrodes are close together (Fig. 4*a*) but also when they are

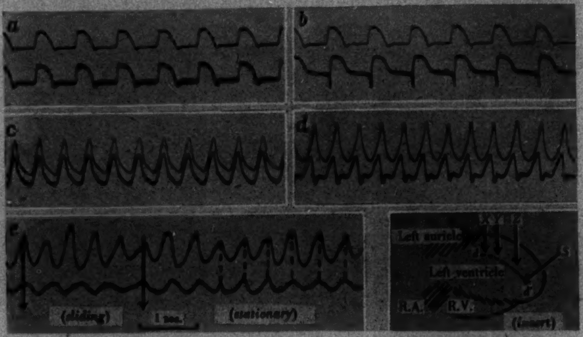


Fig. 4. In all five strips the upper of the two simultaneously recorded electrograms is that taken from a fixed electrode (at position *X* in the *insert*) on the left ventricular surface. The left leg serves as the neutral electrode in all cases. (*a*, *b*) Untreated dog. The lower electrogram has been taken from points *Y* and *Z* respectively. $XY = 1{\cdot}2$ cm. $XZ = 4{\cdot}5$ cm. Even when the direct leads are well apart (*b*), the deflexions recorded are practically in phase. (*c*, *d*) Same dog after ventricular flutter has been induced with amarin and adrenaline. Leads *X* and *Y* were used for (*c*), *X* and *Z* for (*d*). Both pairs of electrograms show the same regular undulation but the curves recorded from the more widely separated pair of electrodes (*X*, *Z*) are appreciably out of phase. (*e*) Also taken during ventricular flutter. In this case the lower electrogram was taken from a movable electrode (*S*) which was slid away from *X* in the direction *d*, *d′*. The visual impression of 'multiple circus movement' is borne out by the fact that, as the distance between electrodes *X* and *S* is increased, the oscillations not only go out of phase, which would be expected if only one wave of contraction were in circulation, but come back into phase again. Presumably at the positions denoted by the arrows in strip *e*, the distance between the electrodes has become equal to that between successive wave fronts.

separated (Fig. 4*b*). During ventricular flutter, however, the wave of excitation does not invade the heart as a whole simultaneously because, when the pairs of electrodes are well separated, the resulting traces are out of phase (Fig. 4*d*), whereas when the electrodes are close together the electrograms are in phase (Fig. 4*c*). During an episode of ventricular flutter, one of these electrodes was slid over the ventricular surface in a direction at right angles to the wave front. The distance between the sliding and the fixed electrode could thus be varied considerably. In this way it was found that the two undulatory waves recorded during flutter go out of phase and then back in again as the moving electrode moves away from the fixed one (Fig. 4*e*). The only reasonable explanation of this finding is that there are two or more waves of contraction sweeping over the ventricles at one and the same time: for the curves could then be approximately in phase not only when the electrodes are close together but also when the distance between them is made equal to the distance between successive wave fronts.

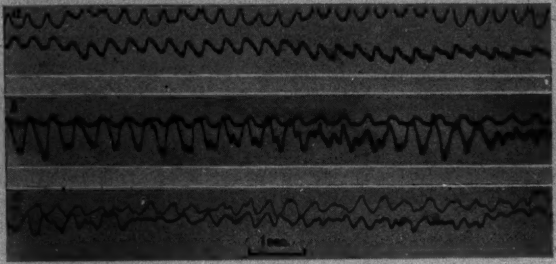


Fig. 5. Break-up of flutter movement. (*a*) Later part of experiment illustrated in Fig. 4*e*. The large steady undulation noted at the beginning of flutter is still a feature of the two simultaneously recorded electrograms, but the lower one especially shows additional cycles superimposed upon this. (*b*) The upper curve is an electrogram led off from near the ventricular septum, the lower is a lead 3 electrocardiogram. This strip was recorded towards the end of an experiment in which ventricular fibrillation was induced in a 17 kg. dog when adrenaline (100 μg.) was injected after a 100 mg. dose of amarin. As flutter changes to fibrillation, the regular undulation becomes less apparent in the electrocardiogram because of the appearance of smaller additional cycles. (*c*) A later stage, showing well-developed ventricular fibrillation.

In a further series of experiments with the same oscillograph an electrogram and an electrocardiogram (lead 2 or lead 3) were recorded simultaneously. The pair of curves so obtained during an episode of flutter are not greatly dissimilar. Both approximate to undulatory curves which are of the same periodicity though commonly out of phase. But, sooner or later, additional cycles creep into the electrocardiogram (Fig. 5*b*) or even into one or two electrograms (Fig. 5*a*). It may be supposed that during flutter the curves are iso-rhythmic because they are both manifestations of the one disturbance; the one series of waves sweeping over the ventricular surface involves both leads though at different times. At this stage, however, comparatively slight changes in the refractory periods of individual fibres should suffice to change the direction of part of an advancing wave front; and if a new, smaller circuit can become established in this manner, irregular re-entry may be expected to proceed apace as a result of the area involved in the circuit deflecting and ultimately breaking up the larger flutter waves. In this way ventricular flutter will be gradually transformed into ventricular fibrillation.

Termination of undulatory movement. In the dog, spontaneous cessation of ventricular flutter was observed on only five occasions in more than eighty experiments. As a rule, ventricular flutter lasted 1 or 2 min. and then changed gradually into ventricular fibrillation, from which condition spontaneous recovery was never observed. Resuscitative measures were often effective, however, particularly the 'serial defibrillation' advocated by Wiggers (1940*a*).

In the cat, flutter movements often ceased spontaneously and without warning. Occasionally, resumption of normal rhythm was preceded by a short standstill of the heart, the 'post-undulatory pause' of Gewin (1906). In other instances the flutter changed into fibrillation as in the dog.

Ventricular fibrillation. The disorder which we, following the terminology of Lewis (1925) and Rothberger (1931), describe as 'ventricular fibrillation' is characterized by the absence of any apparent system or regularity either in the gross visual appearance of the heart or in the electrocardiographic picture; it is a state from which spontaneous return to an effective rhythm must be most exceptional. The distinction between flutter and fibrillation was usually evident both on the electrocardiogram and on the heart itself, though intermediate conditions occurred which may be described as 'flutter-fibrillation' (Fig. 5), and it seems desirable to use distinct terms for the different stages of arrhythmia.

The appearance of ventricular fibrillation after amarin is that of many small wavelets running in different directions, though small circus movements, which are neither long in duration nor constant in position, may be observed occasionally on the heart's surface. In some instances at least, individual regions exhibit a distinct and regular rhythm which is not obvious on simple inspection. This we have been able to detect both from electrograms (Fig. 5) and by the observation of slow-motion films. These films reveal that many regions of the ventricles exhibit regular cycles of mechanical movement. The movements are not necessarily repeated in the same direction nor are they of equal strength. Nevertheless, if the same section of film is run over several times, the periodicity of the rhythms originating in different parts of the ventricles can be ascertained. It has thus been found in some, but by no means all, experiments that the separate regions (delineated by coronary vessels) beat with approximately constant frequencies, although they are not, of course, beating in time with one another. It is especially interesting to note that the rate of contraction of a small region may remain practically constant throughout the transition from ventricular flutter to ventricular fibrillation. Possibly there are numerous foci throughout the heart which discharge impulses at a uniform rate; but it seems more likely that the fairly even rate of beating represents the maximum rate at which heart muscle fibres (under amarin) are capable of contracting in response to excitatory waves.

'Single' circus movement in the ventricles after amarin. The arrhythmia which was usually obtained in the above experiments and which we call 'ventricular flutter', because of its undoubted resemblance electrocardiographically to the clinical disorder referred to by Lewis (1925) as 'ventricular flutter', clearly involves two or more contractile waves, probably circus waves, in progress at one and the same time.

Now Lewis (1925) stated, on electrocardiographic evidence, that ventricular flutter is almost certainly due to a circus movement—i.e. to a wave of excitation in continuous circulation round the ventricles. It is evident from his writings, however, that the rhythm to which he was referring is 'single' circus movement.

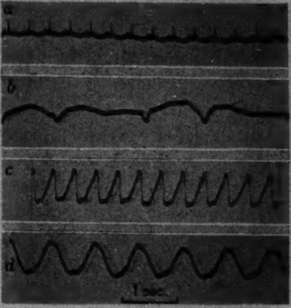


Fig. 6. Cat: 4 mg., 1·5 g. sodium barbitone. Vagi intact. All electrocardiograms taken on lead 2 except *a* (lead 3). (*a*) Control. (*b*) A rhythm of ventricular origin, after amarin, associated with bradycardia and slow contraction of the ventricular muscle. Electrical activity may persist throughout the cardiac cycle. (*c*) A somewhat faster rhythm of ventricular origin. (*d*) Circus rhythm associated with a single wave of contraction coursing slowly and continuously round the heart.

Some interest may attach therefore to the production, in four experiments with amarin, of a ventricular rhythm which was undoubtedly a 'single' circus movement of the type postulated by Lewis but which, nevertheless, gave a different electrocardiographic picture from that which the present authors and almost all previous writers regard as characteristic of ventricular flutter. The slow rate of passage of the contractile wave over the heart's surface made visual observation simple and it was evident to several observers that there was but a single wave coursing steadily round and round the ventricles. It was fortunately possible to take electrocardiograms in the rare instances in which this 'single' circus movement was observed visually. The electrocardiographic picture differs from that of ventricular flutter in one very important respect, viz. the rate of undulation is much less (Fig. 6*d*). Whereas the duration of each cycle of the 'single' circus movement is approximately equal to that of an ordinary ventricular systole following amarin, the duration of an electrocardiographic cycle during clinical or experimental (amarin-induced) ventricular flutter is only a half or a third of this.

This single circus movement was initiated on three occasions towards the conclusion of an experiment by applying faradic stimuli to two parts of the heart in rapid succession. Its onset was thought to be assisted in one case by unequal warming of the surface of the heart with saline pads, which were applied in the hope that local differences in rate of transmission and refractory period sufficient to initiate circus rhythm would be so obtained. In two of these experiments, it may be added, the dogs had been given enough curare to stop contractions of voluntary muscles which were interfering with the electrocardiogram; the amount of curare was unrecorded, its source and purity are unknown. Despite many attempts, the phenomenon could be obtained only exceptionally and in hearts that were in a poor state by this stage of the experiment; conditions under which it can be obtained regularly remain undefined.

A 'single' circus rhythm, which developed more or less spontaneously so far as could be judged, was seen more often in an auricular appendage. It was seen so frequently indeed that it was regarded almost as a feature of experiments in which ventricular fibrillation was induced in anaesthetized dogs with the aid of amarin and adrenaline. As a rule, this arrhythmia did not persist for more than a minute or two.

DISCUSSION

Both clinically and experimentally, there are encountered two allied forms of sudden arrest of cardiac function described respectively as ventricular flutter and ventricular fibrillation.

Flutter can be distinguished from fibrillation in that the electrocardiographic deflexions of flutter resemble each other much more closely. Thus, whereas the electrocardiogram of ventricular flutter approximates to a sine curve, there is little indication of a basic pattern in the smaller bizarre deflexions of ventricular fibrillation.

It has often been suggested that the disorder of rhythm which gives rise to the undulatory electrocardiogram of flutter is a form of re-entry. While hypotheses differ in detail, most proponents of this view have clearly indicated their belief that only one wave of contraction is associated with a given circus.

We think it of interest, therefore, that in experiments with amarin there can be obtained an idio-ventricular rhythm which we feel justified in describing as 'ventricular flutter' from its electrocardiographic appearance and yet which seems to be the result of 'multiple' as distinct from 'single' circus movement.

The abnormally slow manner in which a wave of contraction spreads over the myocardium after the administration of amarin helped us to arrive at this conclusion. Even without the aid of cinematography, we have often been able to observe quite clearly that the 'flutter' movements of the ventricles obtained after suitable treatment with amarin and adrenaline consist of a train of contractile waves which, for appreciable periods, follow out approximately the same course over the ventricular surface, the distance between successive waves usually being so short that two or more can be seen in progress at any one time.

The sliding electrode experiment described above also indicates the presence of several waves of contraction in progress simultaneously, even if it does not show by itself that they are part of circus movement.

Actually, the most striking evidence of circus movement in the ventricles was obtained on the four occasions in some eighty experiments in which but a solitary wave of contraction could be seen to traverse the chambers continuously. This wave coursed round and round the ventricles at a rate sufficiently slow (about one circuit per second) for it to be followed without the least difficulty throughout its entire course. The corresponding electrocardiogram was found to resemble that of ventricular flutter except that the rate of oscillation is much less (cf. Figs. 2*b* and 6*d*). The difference between the two electrocardiograms appears to be due simply to the fact that in flutter (after amarin) there are several waves in the circuit. This causes an increase in the frequency of electrocardiographic cycles, since each circuit of the solitary circus wave has been found to correspond with one electrocardiographic cycle. Our experimental evidence suggests that the electrocardiogram of ventricular flutter as it occurs clinically could be explained by either a 'single' circus wave travelling at a fast rate or 'multiple' circus waves travelling at a correspondingly slower rate. But of the rhythms obtained with amarin, the latter with its rapidly repeated electrocardiographic cycles bears by far the closer resemblance to clinical ventricular flutter.

When ventricular flutter develops after the injection of adrenaline in dogs or cats that have been treated with amarin, the last few cycles of the electrocardiogram obtained before flutter supervenes show chaotic rhythm. They often present an additional striking abnormality, viz. the interruption of the *T* wave of the electrocardiogram by the initial part of the succeeding ventricular complex. The phenomenon may be regarded as an extreme instance of the increase in the duration of ventricular systole relative to that of diastole noted by Chenoweth (1946) in comparable experiments in which he induced ventricular fibrillation by giving adrenaline in conjunction with various hydrocarbons.

This appearance of *R* waves on *T* waves seems to be a frequent, and is possibly a usual, precursor of ventricular flutter or fibrillation. As well as in the above experiments with amarin, the phenomenon has been noted as a rule in experiments in which furfurin or benzene was used in place of amarin for disposing the ventricles towards fibrillation with adrenaline (Fastier, unpublished results). It can be noted, too, in some of the published electrocardiograms illustrating the onset of ventricular fibrillation after the injection of adrenaline in animals treated previously with chloroform (Levy & Lewis, 1911; Melville, 1946), various hydrocarbons (Meek, 1941; Chenoweth, 1946) or

'D.D.T.' (Philips, Gilman & Crescitelli, 1946). It may be added in this connexion that one of us (F.H.S.) has made observations on 17 patients in whom *R* waves were at times to be found superimposed on the antecedent *T* waves: several of these patients have died suddenly.

Even amongst miscellaneous published electrocardiograms we have discovered many examples of *R* waves interrupting *T* waves. With the following exception, we have not as yet found any corresponding comment to indicate that the existence of the phenomenon has been recognized. In a footnote to a review on the *T* wave, Katz (1928) states that one example of an *R* wave on a *T* wave has been observed by Dr Ashman; three others in man have been observed by Drs Feil and Seigal and three by himself; there is one in a published record by Wenchebach & Winterberg; while Wiggers has seen it in dogs.

There seems to be no reasonable alternative to the view that an *R* wave superimposed upon a *T* wave represents the beginning of a new wave of excitation before the previous wave of excitation has ended.

In conclusion we may remark that once re-entry in the guise of the 'multiple circus rhythm' of flutter has come about, it is not difficult to understand how fibrillation develops. As is generally considered, the change from flutter to fibrillation seems to be one of increasing disorganization of contractile units, which are broken down into smaller and smaller aggregates (Wiggers, 1940*b*). Such a progressive disorganization appears manifest in the records which we have obtained from dogs during the transition from flutter to fine fibrillation and which we have described earlier in the paper.

SUMMARY

1. An account has been given of some of the circulatory effects of amarin, a cyclic amidine derivative.

2. Amarin causes profound bradycardia. It lengthens the *PR* interval and prolongs the duration of the *QRST* complex, sometimes to two or three times the normal.

3. After treatment with amarin, the rhythm of the heart may take its origin from the auriculo-ventricular node or from a ventricular focus. In addition, the following disturbances have been observed: partial heart block, branch bundle block, complete heart block, left and right auricles beating independently, ventricles driving the auricles, very large *T* wave, elevation and depression of the *S–T* segment, electrical alternation of the heart.

4. Amarin depresses the special conducting tissue of the heart, but it is not known whether there is an independent effect upon the spread of excitation through heart muscle.

5. Small doses of amarin elevate the blood pressure through peripheral vasoconstriction. Large doses lower the blood pressure, in part at least, through cardiac inhibition.

6. After amarin, the pressor responses to *small* doses of adrenaline are much increased in anaesthetized dogs.

7. *Moderate* doses of adrenaline cause precipitous falls of blood pressure in anaesthetized dogs and cats that have been treated previously with amarin, due to the development of ventricular flutter.

8. In the course of ventricular flutter so induced, deep peristalsis-like waves of contraction occur, at least two such waves usually being visible at the same time on the anterior surface of the heart.

9. In some experiments there was evidence that, before the development of flutter, a new wave of excitation may start in the ventricles before the preceding ventricular wave of excitation has ended. In most instances, flutter followed soon after this phenomenon was observed.

10. Ventricular flutter induced by amarin and adrenaline is due, probably, to 'multiple' circus rhythm; but fully developed ventricular fibrillation, when induced in this way, does not appear to depend upon any simple system of circulating waves of excitation.

The expenses of this research have been defrayed in part by a grant from the New Zealand Medical Research Council. We are also indebted to Prof. R. B. Dodds and Mr R. F. Riggs for taking cinematograph films on our behalf and to Messrs W. B. Johnson, K. T. Fowler, and C. S. W. Reid, for the construction of electrical recording apparatus.

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THE OXIDATION OF HAEMOGLOBIN TO METHAEMOGLOBIN BY OXYGEN

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In an investigation of the oxidation of haemoglobin to methaemoglobin by oxygen (Brooks, 1931, 1935) it was found that (*a*) under constant conditions of oxygen pressure, pH, salt concentration and temperature the reaction was unimolecular with respect to the concentration of haemoglobin; (*b*) the rate of oxidation had a well-defined maximum value at a low pressure of oxygen (19·7 mm. Hg at 30° C.) and over a wide range of pressures could be represented by (1). Haemoglobin includes both oxyhaemoglobin and reduced haemoglobin, i.e. the concentration is proportional to the total number of ferrohaems present.

$$\frac{dx}{dt}=k'm\,(a-x)\,\frac{p}{1+bp}, \qquad (1)$$

where k' and b are constants (0·0230 and 0·0118), m = fraction of haemoglobin in the form of reduced haemoglobin at pressure p, and $a-x$ = percentage of unchanged haemoglobin ($a=100$) at time t.

The rate therefore was proportional to the concentration of reduced haemoglobin $m\,(a-x)$, i.e. to the number of unoxygenated ferrohaems, and to a function of the oxygen pressure. At a constant pressure of oxygen the equation becomes

$$\frac{dx}{dt}=k_1\,(a-x). \qquad (2)$$

It was shown that the rate-determining step was not the selective reaction of oxygen with one or more of the intermediate compounds present (Hb_4, Hb_4O_2, etc.). The concentration of the intermediates was obtained from Adair's (1925) formulation of the equilibrium between haemoglobin and oxygen. It was concluded (*a*) that the unoxygenated ferrohaems in the different intermediate compounds react at the same rate with oxygen, the rate being proportional to the first power of the oxygen pressure, and (*b*) that oxygen also acts as an inhibitor (as in several other reactions), the inhibitory effect being proportional to $1/(1+bp)$.

As the fraction m decreases with increasing oxygen pressure, a maximum rate of oxidation is obtained. It should be noted that the establishment of equilibrium between oxygen and haemoglobin is very rapid in comparison with the rate of oxidation to the ferri-compound methaemoglobin.

Lemberg, Legge & Lockwood (1941) and Legge (1942) have criticized the above mechanism. They consider it unlikely that an unoxygenated ferrohaem can react with oxygen in two different ways, on the one hand to form the addition compound or on the other to be oxidized to the ferri-state. This objection is difficult to understand. The two reactions have very different energies of activation and, relatively, the energy barrier to the second reaction will be surmounted only occasionally. Legge has suggested an alternative which, if valid, offers a simpler explanation of the complex relation between rate and oxygen pressure. Legge concludes that the rate-determining step is the spontaneous breakdown of the intermediate Hb_4O_4 to methaemoglobin; hence

$$k_1 = k_2\,[Hb_4O_4], \tag{3}$$

where k_2 should be a constant independent of the oxygen pressure.

The concentration of the intermediate can be calculated as a fraction of the total concentration of haemoglobin with sufficient accuracy for the present purpose from the experimentally determined oxygen-dissociation curve (Brooks, 1935). Either the original equations of Adair can be used or (as Legge has done) those of Pauling (1935) in which, by assuming interaction between adjacent ferrohaems in the haemoglobin molecule, the four constants are reduced to two. As will be seen from columns 5 and 6 of Table 1, both methods give similar results.

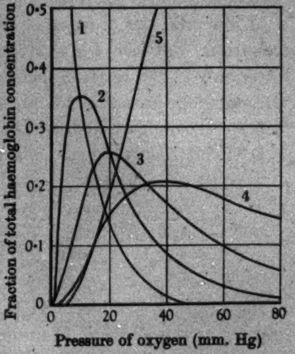

Fig. 1. Relative concentrations of the intermediate compounds of haemoglobin. 1, Hb_4; 2, Hb_4O_2; 3, Hb_4O_4; 4, Hb_4O_6; 5, Hb_4O_8.

Fig. 1 shows the relative concentrations of the intermediates at different oxygen pressures. It will be seen that the concentration of Hb_4O_4 reaches a maximum at 20 mm. Hg O_2 pressure; this is also the pressure at which the rate of oxidation is maximal. Legge calculated values of k_2 only up to an oxygen pressure of 63 mm. Hg. These values are given in the seventh column of Table 1; similar calculations have now been made for pressures between 63 and 723 mm. Hg and are given in the same column.

Even below 63 mm. Hg O_2 pressure the variation in the value of k_2 is greater than can be explained by experimental errors in the values of k_1 and m. At pressures above 63 mm. Hg k_2 increases rapidly (cf. the values of k' from (1) given in the fourth column). In other words, the concentration of Hb_4O_4 decreases much more rapidly than the rate of oxidation: for example the ratio of the concentrations at 19·7 and 723 mm. Hg is 230 whereas the ratio of the rates is 3·9. It is concluded therefore that the breakdown of Hb_4O_4 is not the rate-determining step. The conclusion does not depend on the particular method used to calculate $[Hb_4O_4]$, which must necessarily be very small at high pressures of oxygen.

TABLE 1. The relation between oxygen pressure and the rate of oxidation of haemoglobin

p (mm. Hg)	$k_1 \times 10^2$	m	$k' \times 10^3$	$[Hb_4O_4]$ (Adair)	$[Hb_4O_4]$ (Pauling)	$k_2 \times 10^3$
4·5	9·9	0·89	2·61	0·065	0·060	165
6·0	12·6	0·85	2·64	0·099	0·094	134
13·7	15·9	0·64	2·11	0·232	0·227	70
16·0	17·6	0·57	2·29	0·249	0·243	72
25·8	17·7	0·39	2·29	0·239	0·232	76
33·3	14·5	0·29	2·09	0·200	0·195	74
63·1	10·3	0·12	2·37	0·089	0·090	114
92·9	8·45	0·08	2·38	0·047	0·049	173
122·0	7·40	0·06	2·47	0·029	0·031	239
152·0	6·36	0·05	2·34	0·019	0·021	303
435·0	5·17	0·03	2·43	0·0026	0·0027	1890
723·0	4·61	0·03	2·03	0·00094	0·0011	4190

At low pressures of oxygen $[Hb_4O_4]$ increases more rapidly than the rate of oxidation. Between 4·5 and 6 mm. Hg the former increase is 57 % and the latter only 27 %. In this range, equation (1) indicates approximately that the rate should be proportional only to the first power of the oxygen pressure, since bp is small compared with unity and there is only a small variation in the value of m; Table 2 shows that this is the case.

TABLE 2. The relation between oxygen pressure and the rate of oxidation at low pressures of oxygen

p (mm. Hg)	m	$k_1 \times 10^2$	k_1/p	$[Hb_4O_4]$
4·5	0·89	9·9	0·022	0·060
6·0	0·85	12·6	0·021	0·094

The pressure at which $[Hb_4O_4]$ is greatest is, however, not without significance; at this pressure the number of oxygenated and unoxygenated ferrohaems is equal, i.e. $m = 0·5$ (cf. Corryell, Pauling & Dodson, 1939). It can be shown from equation (1) (by expressing m in terms of p) that, since bp is not large when the oxygen pressure is about 20 mm. Hg, the maximum rate occurs when m is practically 0·5.

The oxidation of myoglobin by oxygen

Myoglobin in muscle is oxidized by oxygen and the rate also reaches a maximum at a low pressure of oxygen (Brooks, 1929). As myoglobin contains only one ferrohaem (Svedberg, 1938) there can be no question of intermediate compounds and the rate-determining step cannot be the spontaneous decomposition of MbO_2, else the rate would increase with increasing oxygen pressure. It would seem therefore that reduced myoglobin reacts either with oxygen or with oxymyoglobin. The latter reaction would be bimolecular with respect to the concentration of myoglobin and above about 1 mm. Hg O_2 pressure the rate would be inversely proportional to the pressure. The order of the reaction is unknown but the available data, although only semi-quantitative, indicate a smaller variation of rate with pressure, of the same order as that found in the case of haemoglobin.

SUMMARY

The rate of oxidation of haemoglobin to methaemoglobin by oxygen is greatest at a low pressure of oxygen. Legge has pointed out that, at the same pressure, the concentration of the intermediate Hb_4O_4 also reaches a maximum and suggests that the rate-determining step is the spontaneous decomposition of Hb_4O_4. It is shown that at an oxygen pressure above about 90 mm. Hg the concentration of Hb_4O_4 decreases much more rapidly than the observed rate of oxidation, and the suggested mechanism is therefore believed to be invalid.

The work described above was carried out as part of the programme of the Food Investigation Board. It is published by permission of the Department of Scientific and Industrial Research.

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OBSERVATIONS ON THE URINE, BLOOD AND ARTERIAL PRESSURE OF DOGS BEFORE AND AFTER THE PRODUCTION OF RENAL ISCHAEMIA*

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Lockett (1946) describes the appearance of a new base, detected by a colour test and indicated as x-positive base (x), in the urine of bitches after partial obstruction of the renal artery, this base not being present in normal dog or human urine. x was measured by Richter's (1938) method in terms of ephedrine HCl, solutions of which after addition of NaOH and extraction with toluene give a colour with picric acid in chloroform indistinguishable from that given by the urine when similarly treated; and the amount of x was expressed as ephedrine HCl equivalent (E.E.). Lockett found that in each of seven normal bitches in which the right renal artery was partially obstructed, the urine was x-negative before operation, but when re-examined 4 or 5 days after operation it was x-positive and remained so throughout the 3 months of observation, and that control examinations of the urine of 4 bitches undergoing other operations yielded negative results.

Lockett further reports that the new base appears in the urine within 10 min. of the compression of one renal artery and continues to be excreted for some hours after the release of compression, that it is excreted mainly by the normal kidney, that the blood becomes x-positive during the compression, and that the colour test is given by saline extracts of normal renal cortex but by none of sixteen other tissue extracts which were examined. Finally, Lockett describes the results of long-term observations on 2 dogs in which partial obstruction of one renal artery was followed at a later stage by partial obstruction of the other: after the first operation the blood and urine became x-positive, and as the blood pressure fell x disappeared from the blood but persisted in the urine; and after the second operation the rise in arterial pressure was again associated with the appearance of x-positive base in the blood. When the blood pressure was raised by limitation of fluid intake or by the administration of simple salts, the *x*-content of the blood rose and the rate of excretion of *x* in the urine fell, these results being reversible. In the production of these changes the potassium ion is claimed to be more effective than the sodium ion, and the chloride than the acetate ion.

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* An abstract of this work was communicated at the 17th International Physiological Congress, Oxford, in July 1947.

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Lockett's results seemed to us of such importance as to merit a repetition of those experiments on which her primary findings are based. We have been unable to confirm them.

METHODS

Estimation of x in the urine. Urine samples were treated as described by Lockett (1946); the urine was, however, always filtered, and after the solution of NaOH and the toluene had been added, the fluid was shaken mechanically for 10 min. Each sample of urine was usually 10 c.c.; when not, it was made up to this volume with distilled water. The toluene and chloroform were purified by washing, drying over $CaCl_2$ and re-distilling (110–111° and 61–62° C. respectively). 1·5 c.c. purified toluene and 1·5 c.c. purified chloroform gave, when 0·1 c.c. picric acid, 2% in chloroform, was added to the mixture, a very pale green-yellow colour: this was evaluated in each series of estimations and subtracted from the value of the mixture of 1·5 c.c. toluene extract of urine, 1·5 c.c. chloroform and 0·1 c.c. of the picric acid reagent. Very seldom the upper toluene layer, after centrifuging, was itself slightly greenish: in that event, after the addition of an equal volume of chloroform, the colour was compared with that of the diluted ephedrine standard (see below) and its value also subtracted from that obtained after adding the picric acid reagent to equal volumes of chloroform and the toluene extract of urine. When the animal's diet was milk and biscuit only, the upper toluene layer was found almost invariably to be colourless. Duplicate extractions gave E.E. values which differed usually by 10%, occasionally 30%; but when the urine values were very low, e.g. $0{\cdot}5 \times 10^{-6}$ g. E.E./c.c., this difference might be 50%: little quantitative significance is to be attached to values below this.

Estimation of x in the blood. 20 c.c. of arterial blood—obtained by puncture of a carotid loop (Verney & Vogt, 1938)—or of venous blood were withdrawn into a syringe containing 1 mg. heparin in 1 c.c. 0·9% NaCl, and mixed with 4 c.c. 10% NaOH and 6 c.c. purified toluene. The mixture was shaken by circular motion of the hand for 15 min., and centrifuged for 15 min. 1·5 c.c. of the upper toluene layer—this was invariably colourless—were then treated and examined in the same way as a toluene extract of urine.

Ephedrine standard. 0·1 g. ephedrine HCl in 50 c.c. distilled water with 10 c.c. 10% NaOH were shaken mechanically with 50 c.c. purified toluene for 10 min. and centrifuged for 15 min. The toluene layer was again centrifuged for 15 min., and the upper 30 c.c. were withdrawn and stored in a refrigerator. 1·5 c.c. of this toluene extract with 1·5 c.c. chloroform and 0·1 c.c. picric acid, 2% in chloroform, gave the colour standard, and this was freshly prepared for each series of determinations: the corresponding ephedrine HCl content of this standard was defined as 10^{-4} g./c.c. For colorimetric estimations of the E.E. of toluene extracts of urine and blood, this standard was diluted 1:10 with purified toluene.

Collection of urine. The bitches had been perineotomized and trained to lie at rest on a warmed table. In short-term observations on the effects of partial obstruction of a renal artery the urine was collected by catheter over successive periods which varied between 4 and 20 min. In the long-term experiments two methods were used for the determination of the hourly urine and *x*-outputs. In the one, the bladder was emptied by catheter in the morning; the dog was then put into a metabolism cage, and 1–5 hr. later the bladder was again emptied by catheter. In the other, the dogs were kept in metabolism cages during successive periods of 24 hr. with the exception of the half-hour immediately following the morning catheterization. The urine passed in the metabolism cages was collected in clean and toluened glass jars. Its volume was measured, and its *x*-content determined; the morning catheter sample was similarly treated. We shall refer to these two methods as the short-term process (S) and the long-term process (L) respectively. Lockett (1946) used metabolism cages when the total output for the day was required.

Diet. The standard diet was 8 oz. biscuit and 300–500 c.c. milk. Meat (4–16 oz. daily) was given during recovery from surgical procedures, and at certain other times to which reference will be made in the description of the experimental results. Water was usually allowed *ad lib.*; sometimes, however, it was limited; and sometimes measured volumes were given by stomach tube.

Partial obstruction of the renal arteries. In the short-term experiments this was achieved by compression of a renal artery loop (Lockett, O'Connor & Verney, 1942) by means of a small adjustable clamp which was slipped around the loop: the pressure on this could be nicely varied by two screws which governed the movement of the compression plate of the clamp. In the long-term experiments the renal arteries were compressed by silver clips implanted by the method described by Verney & Vogt (1938).

Measurement of the systolic arterial pressure. The carotid loop with denervated sinus was used, the surgical procedures and technique of measurement being those described by Verney & Vogt (1938).

RESULTS

The presence of x in the urine and blood of normal dogs

We investigated repeatedly the urine of 6 perineotomized bitches whose kidneys had not been interfered with in any way. In every instance the urine was x-positive, and the x-content varied within the wide limits of 1 and 29×10^{-6} g. E.E./c.c. (4 animals, standard diet with meat supplement) and of 0·8 and 55×10^{-6} g. E.E./c.c. (4 animals, standard diet). Even on the same day, if the dogs were catheterized twice, and these samples together with the 24 hr. specimen were investigated, their x-values/c.c. might be very different (see Fig. 1, first part). To 2 animals water only was allowed for a period of 51 hr.: the urine remained, nevertheless, x-positive during the fast, the x-content varying between 2·6 and $19{\cdot}6\times10^{-6}$ g. E.E./c.c. No clear relation was to be found between x-content and specific gravity. Further, we observed that extraction of a single specimen of urine at different times after its collection by catheter sometimes gave x-values which differed by more than the error of the colorimetric determinations, but no clear relation between change in x-content and time was to be found. To minimize such change the specimens of catheter urine were always extracted within an hour of their having been obtained. The x-content of the 24 hr. specimens of urine of the animals in metabolism cages was determined each morning.

The hourly x-output with standard diet and meat supplement lay, by the S process, between 2·0 and $2{\cdot}8\times10^{-4}$ g. E.E. (mean 2·4, 2 observations, 2 animals) and, by the L process, between 0·1 and $3{\cdot}2\times10^{-4}$ g. E.E. (mean 0·9, 25 observations, 2 animals). With standard diet it lay, by the S process, between 0·1 and $1{\cdot}5\times10^{-4}$ g. E.E. (mean 0·7, 17 observations, 4 animals) and, by the L process, between 0·3 and $4{\cdot}4\times10^{-4}$ g. (mean 1·0, 24 observations, 2 animals); and on 2 fasting animals it lay by the S process between 0·3 and $2{\cdot}4\times10^{-4}$ g. E.E. (mean 1·1, 4 observations). The differences in the hourly

x-output by the S and L processes did not as a rule exceed the ratio 1:2. Sometimes the S values were the higher, sometimes the L values.

The blood of normal dogs (3 animals, 6 specimens) was found in each instance to be faintly x-positive, and colorimetric estimation gave values of 0·3, 0·4 and $0{\cdot}4\times10^{-6}$ g. E.E./c.c. (standard diet) and 0·3, 1·0 and $2{\cdot}1\times10^{-6}$ g. E.E./c.c. (standard diet with meat supplement). While little quantitative reliance can be placed on such low values, we were unable, as will be seen later, to detect any definite change as the result of renal ischaemia.

Long-term experiments on obstruction of the renal arterial supply and x in the urine and blood

Although we were unable to confirm Lockett's x-negativity of the urine of normal bitches, we decided, nevertheless, to see whether we could detect any increase in x-output as the result of renal ischaemia.

Two animals, J and W, were used for these experiments, and the results obtained with one of them (J) are given in Fig. 1. For 8 weeks before the first operation (partial obstruction of the right renal artery) each and every examination of the urine and blood (arterial and venous) gave an x-positive result, and the fluctuations in the x-content of the urine and in the hourly output of x are illustrated in the figure. The hourly output of x (standard diet) lay, by the S process, between 0·1 and $1{\cdot}5\times10^{-4}$ g. E.E. (mean 0·6, 10 observations), and, by the L process, between 0·3 and $4{\cdot}4\times10^{-4}$ g. E.E. (mean 1·1, 19 observations). The systolic B.P. was often measured: it lay between 115 and 120 mm. Hg. After the right renal artery had been partially obstructed (R, Fig. 1), the B.P. rose to between 154 and 158 mm. Hg, at which level it was maintained up to the time of the second operation. During this period, however, no clear change in the hourly x-output was observed—the values (standard diet) lay, by the S process, between 0·2 and $1{\cdot}8\times10^{-4}$ g. E.E. (mean 0·9, 9 observations), and, by the L process, between 0·2 and $2{\cdot}5\times10^{-4}$ g. E.E. (mean 1·0, 9 observations)—nor was any definite change detected in the x-content of the blood. Four weeks after the obstruction of the right renal artery, the two arteries which supplied the left kidney in this animal were similarly obstructed (L, Fig. 1). As a result of this the B.P. rose to between 182 and 184 mm., but the hourly output of x was as irregular as before: the values (standard diet) lay, by the S process, between 0·1 and $1{\cdot}6\times10^{-4}$ g. E.E. (mean 0·5, 6 observations) and by the L process between 0·1 and $2{\cdot}6\times10^{-4}$ g. E.E. (mean 0·9, 11 observations). Again no change in the x-content of the blood was detected.

Essentially similar results were obtained with the second animal (W). This animal's B.P. was 133 mm. Hg, and its diet was supplemented with 4 oz. meat. The period of observation before partial obstruction of the right renal artery was 1 week only, and, during this, the hourly output of x as determined from estimations on the 24 hr. specimens of urine varied between 0·4 and $1{\cdot}4\times10^{-4}$ g.

E.E. (mean 0·8, 7 observations). After obstruction—the B.P. then having risen to 146–171 mm.—it varied between 0·4 and $1\cdot7\times10^{-4}$ g. E.E. (mean 0·9, 7 observations), and no definite change in the x-content of the blood was

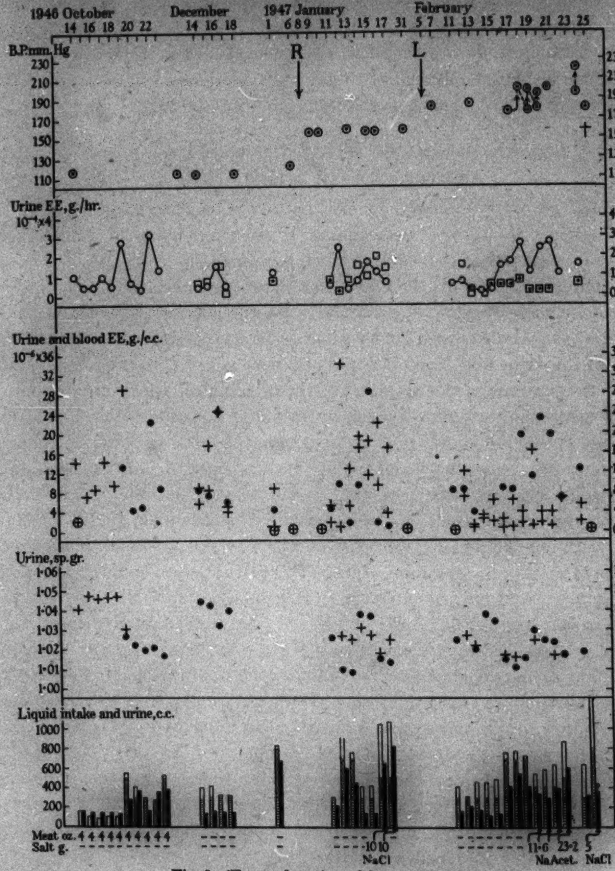

Fig. 1. (For explanation of figure see p. 341.)

detected. Two months after the first operation a renal artery loop was made on the left side: healing was per secundam and partial obstruction of the artery was thereby produced. The B.P. rose to 191 mm. Hg, but no change in the x-content of the blood was detected, and two determinations of the hourly output of x (0·6 and $2{\cdot}0\times10^{-4}$ g. E.E., again by the L process) showed no significant alteration from the previous values: all are within the range of variation encountered in normal animals.

x in the urine and blood during periods of limitation of fluid intake, and after the administration of salts

Lockett (1946) describes an increase in B.P. and in the x-content of blood and a fall in the x-output when, after partial obstruction of one renal artery, the fluid intake was limited to 600 and 800 c.c. daily, these changes being reversed when water was freely given. In Lockett's Fig. 2, some 10 days after the period of limitation, a similar fall and subsequent rise in x-output is recorded without such limitation. We cannot confirm her results. With the dog W we repeatedly limited the fluid intake, and were unable to detect any clear change in the x-output as a result of this procedure. A blood test was made on the third day of one period of limited fluid intake: the x-value was seemingly a little lower than that of a blood sample taken the day before the limitation of fluid intake began. Lockett (1946) also investigated the influence of some salts upon the B.P., the x-content of blood and the x-output, and found that, after partial obstruction of one renal artery, the giving of 10 g. sodium chloride daily for 3 and for 4 days did not alter the B.P., but caused a lowered hourly output of x in one experiment, and 'may have caused slight reduction' in the other. After partial obstruction of the arterial supply to the contralateral kidney, the administration of 8 g. potassium acetate daily for 3 days was

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Fig. 1. The influence of partial obstruction of the renal artery on one and on both sides of the dog J upon the excretion of x. See also text. The figure shows only a few and typical findings extracted from those obtained during the period of observation. The liquid intake and urine relate to the 24 hr. ending at about 10 a.m. of the indicated day, and the arrows above the salt dosage at the bottom of the figure lead from the days of administration to the corresponding 24 hr. collections of urine. When the urine flow was also measured over short intervals, with or without the initial administration of water and salts, the data in connexion with these urine samples are noted on the days on which the observations were made; but the E.E. value of such sample was also taken into account in calculating the E.E. output for the 24 hr. period ending at 10 a.m. of the next day.

▯, water by mouth; ▨, water by stomach tube; ▤, milk by mouth.

▮, urine (cage and catheter urine); ●, cage urine; +, catheter urine.

⊕, blood E.E.; ⊙, blood pressure.

○, the hourly E.E. outputs derived from the cage and catheter urine values over 24 hr. periods; □, the hourly E.E. outputs derived from the urine values over short intervals of 1–5 hr.; ⊡, as □ but following the administration of water (200 c.c., or 100 c.c. + 11·6 g. $NaC_2H_3O_2 3H_2O$, or 100 c.c. + 5 g. NaCl) by stomach tube at the beginning of the short interval.

Arrow R, first operation, i.e. right renal artery partially obstructed by a silver clamp. Arrow L, second operation, i.e. the two branches of the left renal artery partially obstructed by silver clamps.

The administration of meat and of different salts is noted at the bottom of the figure.

accompanied by an increase in B.P. and a fall in x-output. In the author's Fig. 2, however, the x-output is falling rapidly before the potassium acetate is given, and when (author's Fig. 1) a similar fall before and during the administration of 11 g. sodium acetate for each of 3 successive days is shown, the sodium acetate is stated to be without effect. The following of the sodium acetate with 8 g. potassium acetate daily for 3 days is stated to be accompanied by a fall in x-output, a statement which the figure does not substantiate. Lastly, in one of the author's experiments (Fig. 2), potassium chloride was given, 13 g. daily for 2 days and 6·5 g. for 1 day, and the effects are stated to be similar to those of the first administration of potassium acetate. The author's figure shows, however, that by the third day of potassium chloride administration the x-output was only beginning to fall, whereas by the third day of potassium acetate administration the x-output had reached zero.

With our two animals, J and W, we have made a few observations with respect to the effects of sodium chloride and sodium acetate administration on the B.P., blood-x and x-output. In the animal J, before any operative interference with the renal circulation, daily doses of 5, 10 and 10 g. sodium chloride caused no change in B.P. and no uniform response in x-output, this remaining unchanged on the first day, increasing on the second, and decreasing on the third. Examination of the blood on the third day showed no significant change in x-content from that on days when no salt was being given. Eight days after obstruction of the right renal artery, 10 g. sodium chloride were given daily for 2 days (Fig. 1): no change in B.P. occurred, and the hourly output of x showed a slight fall (L process) and an increase followed by a fall (S process), variations such as were also encountered during periods when no salt was being given. Thirteen days after partial obstruction of the left renal artery 11·6 g. sodium acetate ($3H_2O$) were given daily for 3 days. Within 6–8 hr. of the administration of each dose of the salt the B.P. had increased by some 15–20 mm. Hg. On the following day, double the dose of sodium acetate was given. No significant changes in the x-output occurred as the result of these doses of sodium acetate (Fig. 1). Three days afterwards, the B.P. being 195 mm. Hg, 5 g. sodium chloride were given in 100 c.c. water by stomach tube, and 5 hr. later the B.P. had risen to 222 mm. Hg. Over the 75 min. period after the administration of the salt the x-output was not appreciably changed. On the following morning vomiting, haemorrhagic diarrhoea and anuria occurred, and later in the day the animal became semi-conscious: a carotid blood sample was then obtained, the B.P. being 180 mm. Hg, and examination of this blood revealed no increase in x-content. With the other animal (W), 45 days after partial obstruction of the right renal artery, the administration of 10 g. sodium chloride and 20 g. urea daily for 3 days was associated with an increase in x-output on the first day and a decrease on the second and third day, but the changes were no greater than those observed when no salt was being given.

One observation with potassium acetate (8·5 g.) was made on this animal during the period of hypertension following partial obstruction of the contralateral renal artery: if the salt produced any effect at all on the B.P. it was in the direction of fall, because 6 hr. after the salt was given a reading of 178 mm. Hg was obtained, whereas on each of the two previous days the reading was 196 mm. Hg.

Short-term experiments on obstruction of the renal arterial supply and x in the urine and blood

Since we have found that the urine of normal bitches is *x*-positive, we have been unable to test Lockett's claim that a new base, '*x*-positive base', appears in the urine within 10 min. of the production of renal ischaemia. We have, however, made a few observations on the effects of partial and temporary obstruction of the arterial supply to one kidney on the output of *x* in the urine and on the *x*-content of the blood, since we happened to have available an animal in which a renal artery loop had been made on the left side 5 years ago: a year before this the right renal artery had been compressed by a silver clip. A month after the renal artery loop had completely healed the animal's B.P. was 122 mm. Hg. With this animal, observations on the effects of compression of the renal artery loop were made both at high and at low rates of

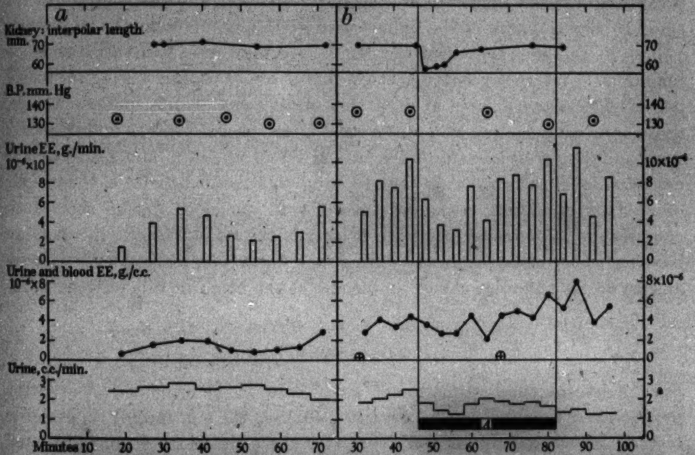

Fig. 2. The excretion of *x* in short-term experiments (*a*) without, and (*b*) with compression of the left renal artery (in a loop) of the dog. See also text. The dog was given at the beginning of the experiment (*a*) 300 c.c. and (*b*) 350 c.c. water by stomach tube. Abscissae, time after the administration of the water.

urine flow. Seven experiments were made: in three of them the renal artery loop was compressed for periods of 36, 36 and 38 min., and in the remaining four no such compression was effected. The periods of observation ranged from 60 to 115 min. The x-output in control periods of urine collection varied between 1·3 and $10{\cdot}4 \times 10^{-6}$ g. E.E./min., and in periods of urine collection during and after compression of the renal artery it varied between 1·7 and $11{\cdot}6 \times 10^{-6}$ g. E.E./min. In one and the same control experiment the x-output might vary by as much as 400%. The results of two experiments which illustrate our findings are given in Fig. 2, those on the left being of a control experiment, those on the right of an experiment in which the renal artery loop was compressed. No change in B.P. and no clear change in the x-content of the blood resulted from the compression. In this experiment the reduction in the interpolar length of the kidney produced by the compression was maintained for some 10 min., the size of the kidney then gradually increasing to its normal value. In another experiment the degree of compression was varied so as to maintain the interpolar length of the kidney at about 60 mm. during the whole period. The average rates of urine flow during the 38 min. periods before, during and after compression of the renal artery were 0·29, 0·19 and 0·28 c.c./min., and the average x-outputs 2·6, 1·7 and $2{\cdot}6 \times 10^{-6}$ g. E.E./min. respectively: compression of the renal artery produced no significant increase in the output of x. Lockett reports the appearance of x in the blood during short-term compression of one renal artery: in neither of the two instances, in which we have tested the blood before and during short-term compression, have we been able to detect any clear change in blood-x as the result of this procedure.

DISCUSSION

For the detection and estimation of 'a new base' in the urine of dogs after the production of renal ischaemia Lockett (1946) used the non-specific colorimetric method introduced by Richter (1938) for the estimation of ephedrine and similar amines in human urine after their administration in large doses by mouth. Lockett applied Richter's test to 140 different samples of normal canine and human urine, reports them free from colour producing compounds, and on the basis of this x-negativity finds x-positive base appearing in the urine and blood after the production of renal ischaemia. We find, on the contrary, that the urine of normal dogs is positive to Richter's test and that the rate of excretion of the substances responsible for this positivity is variable. Moreover, we have been unable to detect any significant change in the output of such substances as the result of renal ischaemia. Neither have we been able to confirm the association described by Lockett between variations in the height of the arterial blood pressure and in the degree of x-positivity of the blood, and between temporary increases in the degree of hypertension following the limitation of fluid intake or the administration of simple salts and temporary increases and decreases respectively in the 'chemical compound x' in the blood and its rate of excretion by the kidney.

It is of interest that v. Euler (1945) has detected piperidine (isolated as picrate) in urine. In dog's urine only small amounts were found ($2–7 \times 10^{-6}$ g./c.c.), whereas in human urine the amounts were higher ($3–20 \times 10^{-6}$ g./c.c.). We have found that aqueous solutions of piperidine when extracted and tested by Richter's method, give a colour like that given by urine when similarly treated, but that 5×10^{-4} g. piperidine/c.c. corresponds colorimetrically with only about $1{\cdot}3 \times 10^{-6}$ g. E.E./c.c. urine. When piperidine in such amount was added to urine the E.E. value of the urine was increased by about the E.E. value of the piperidine alone.

SUMMARY

1. The claims made by Lockett (*a*) that the production of renal ischaemia and hypertension by operative methods is accompanied by the appearance in the blood and urine of a basic compound (detected and estimated by a colour test) which is not present under normal conditions; and (*b*) that clear relations exist between raising the blood pressure, whether by limitation of fluid intake or by administration of simple salts, and a rise of this compound in the blood and a fall in the urine, have not been confirmed.

2. The colour test is positive when applied to the urine of normal dogs, and no significant increase during long-term, or during and after short-term, obstruction of the renal artery has been detected.

3. The hourly output of the substances responsible for the colour test fluctuated, even with a constant diet of milk and biscuit, between 0·1 and $4{\cdot}4 \times 10^{-4}$ g. ephedrine hydrochloride equivalent.

4. Piperidine in such small amounts as have been found in the urine of dogs by v. Euler also gives the colour test.

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THE EFFECT OF DI*ISO*PROPYL FLUOROPHOSPHONATE ON NEUROMUSCULAR TRANSMISSION IN CATS

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In 1942 it was shown that di*iso*propyl fluorophosphonate (D.F.P.) had a much stronger inhibiting action on the cholinesterase of human plasma than eserine (Adrian, Feldberg & Kilby, 1947). When injected intravenously into an animal, however, D.F.P. produces much less muscular twitching and fibrillation than eserine. It appeared, therefore, possible that the action of D.F.P. and eserine on neuromuscular transmission might be different. On the other hand, this difference might be accounted for by the fact that D.F.P. has a greater affinity for the pseudo-cholinesterase, which is present in human plasma, than for the true cholinesterase (Hawkins & Mendel, 1947).

So far only a few experiments have been reported in which the effect of D.F.P. on neuromuscular transmission in mammals has been analysed. Harvey, Jones, Talbot & Grob (1946) injected D.F.P. arterially into men and observed effects very like those seen with eserine, but differing in the predominance of a long-lasting depression. In cats, Riker & Wescoe (1946) observed muscular twitching after arterial injections of D.F.P. and a 'curari-form' effect when acetylcholine was injected after the D.F.P.

A preliminary account of these experiments has already appeared in this *Journal* (Brown, Burns & Feldberg, 1947).

METHODS

The experiments were performed on cats decerebrated under ether anaesthesia. The tibialis anterior muscle was prepared for arterial injection and for stimulation of the nerve by the method described by Brown (1938). The sciatic nerve was stimulated through shielded silver electrodes. The stimulation was provided from an air-core transformer through the primary coil of which a condenser was discharged. For direct stimulation of the muscle, a rectangular wave of voltage (0·5 msec. × 100 V. approx.) was applied between the tendon and the knee joint. In a few experiments the sciatic nerve on one side was divided in the thigh under ether anaesthesia with aseptic precautions and allowed to degenerate for 7 days.

For each experiment a fresh solution of 5–10% D.F.P. in propylene glycol was prepared and diluted with saline solution to the concentration required for injection. For the injections of acetylcholine the chloride was used and the weights given in the text refer to the salt.

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* With a grant from the Medical Research Council.

RESULTS

Fig. 1 illustrates the effect of an arterial injection of 200 μg. D.F.P. upon the tension developed by the tibialis anterior of the cat in response to maximal single motor nerve volleys at 10 sec. intervals. After a latent period of about 1 min. the tension gradually increases and reaches a maximum within 10 min. In this experiment the tension increased from about 0·7 to 1·5 kg.; in some experiments it attained three times the original twitch tension. After having reached its maximum, the tension declines gradually, and, within the next 30–40 min., may fall to below the original twitch tension. With a smaller dose of D.F.P., e.g. 100 μg., the onset of the potentiation of the twitch may be slower; the peak of the potentiation may be reached later, and the subsequent decline

Fig. 1. Tension response of cat's tibialis anterior to maximal nerve volleys every 10 sec. Arterial injection of 200 μg. D.F.P. (first arrow) and of 2·5 μg. acetylcholine (second arrow).

Fig. 2.

Fig. 3.

Fig. 2. Tension response of cat's tibialis anterior to maximal nerve volleys every 10 sec. At arrow, arterial injection of 1 mg. D.F.P.

Fig. 3. Tension response of cat's tibialis anterior to maximal nerve volleys after arterial injection of 100 μg. D.F.P. during the period of depression. Nerve stimulation 1 in 10 sec. interrupted for 1, 2, 5 and 10 min. between *a*, *b*, *c*, *d* and *e* respectively.

may be less pronounced. Doses much under 100μg. have no visible effect on the twitch tension. With doses of the order of 1 mg. there is an almost immediate and very great increase in the tension response and vigorous spontaneous contractions. This potentiation, however, is of short duration, and the tension may rapidly fall to less than before the administration of D.F.P. (Fig. 2).

During the depression of the tension response, some recovery of the tension can be obtained by interruption of the stimulation for 1–10 min. The first response of the subsequent regular series is then considerably greater than those preceding or following it (Fig. 3).

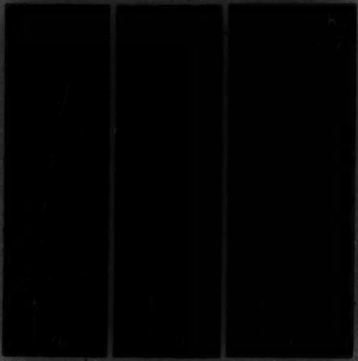

Fig. 4. Effect of 5 sec. tetanus (stimulus-frequency, 100/sec.) on tension response of cat's tibialis anterior to single maximal nerve volleys every 10 sec. (*a*) before and (*b* and *c*) after an arterial injection of 100μg. D.F.P.

Before the administration of D.F.P., stimulation of the motor nerve for 5–10 sec. at frequencies between 50 and 100/sec. (Fig. 4*a*) produces a sustained contraction of the muscle and the subsequent single twitches are increased in tension (cf. Brown & von Euler, 1938). After D.F.P. the muscle no longer maintains its tension during tetanic stimulation, and the responses to subsequent single nerve volleys show a depression from which the muscle recovers almost completely within 1 or 2 min. As shown in Fig. 4 this transient depression occurs not only when the tetanic stimulation is given during the period in which the D.F.P. has augmented the tension response to single motor nerve volleys (Fig. 4*b*), but also during the subsequent period of depressed response (Fig. 4*c*).

Before D.F.P. an arterial injection of 2·5–5μg. of acetylcholine produces a quick 'twitch-like' contraction, the tension of which is usually a little greater than that of a maximal twitch. The acetylcholine contraction may be followed by a transient small increase in the tension of subsequent responses to single motor nerve volleys: this effect is similar to, but less than that following, a brief tetanic stimulation of the nerve. After D.F.P. the quick contraction produced by acetylcholine may be either increased or decreased; if acetylcholine is given during the D.F.P. potentiation it is usually increased; if given during the subsequent depression the response is often much less than that given by the untreated muscle. In both instances, however, the quick response is followed by a profound and often irreversible depression of the response of the muscle to stimulation through its nerve (Figs. 1 and 6). The depression produced by injected acetylcholine thus differs from that produced by tetanic stimulation of the nerve both in its intensity and in its duration.

Fig. 5. Cat, 2 kg. Tension response of tibialis anterior to maximal nerve volleys every 10 sec. At *a*, intravenous injection of 20 mg. D.F.P.; at *b*, 5 sec. tetanus (stimulus-frequency, 50/sec.)

On a number of occasions we have observed that the quick twitch-like contraction resulting from the arterial injection of acetylcholine is followed, in a muscle which has received D.F.P., by a slow and small secondary development of tension similar to, but much smaller than, that occurring in denervated muscle.

In contrast with its effect by arterial injection, D.F.P. given intravenously has a relatively insignificant potentiating effect on the tension responses of the tibialis anterior, and very large doses have to be given to obtain an obvious action. In the experiment of Fig. 5 the intravenous injection of 20 mg. D.F.P. produced, after a latency of some 7 min., only a small potentiation which developed slowly. Under these circumstances, however, the depressing effect of the D.F.P. becomes evident when either acetylcholine is injected arterially or the nerve to the muscle is stimulated for 5–10 sec. at 50–100/sec. The depression so produced by nerve stimulation is of long duration and resembles that produced by arterial injection of acetylcholine into a muscle which had previously received D.F.P. by the arterial route.

The potentiation by D.F.P. of the tension response of the muscle to single maximal motor nerve volleys is due to a repetitive contraction of the muscle fibres. Fig. 6 shows the action potentials of the cat's tibialis anterior taken before (*a*) and at the peak (*b*) of the D.F.P. potentiation of tension. During the potentiation the main action potential is followed by a series of declining potentials. The effect of D.F.P. thus closely resembles that of eserine. Even during the subsequent depression the response remains repetitive; for instance, in Fig. 6*c* the tension response to single nerve volleys was reduced considerably below its former value by an arterial injection of acetylcholine, but the repetitive character of the muscle response continued as shown in the electrical record.

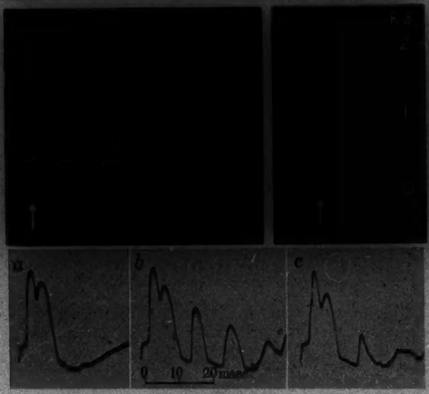

Fig. 6.

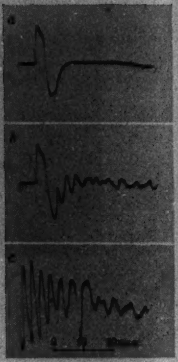

Fig. 7.

Fig. 6. Tension response and action potentials of cat's tibialis anterior to maximal nerve volleys every 10 sec. Arterial injection of 100 μg. D.F.P. (first arrow) and of 5 μg. acetylcholine (second arrow). The action potential *a* was obtained before D.F.P.; that of *b* during the D.F.P. potentiation, and that of *c* during the acetylcholine depression.

Fig. 7. Action potentials recorded from the inferior oblique muscle of the cat on maximal stimulation of the third nerve (*a*) before and (*b*) after arterial injection of 100 μg. D.F.P.; (*c*) shows the action potential recorded during a spontaneous contraction after D.F.P.

In one experiment, the effect of arterially injected D.F.P. was examined on the inferior oblique muscle of the eye, prepared according to the technique described by Brown & Harvey (1941). D.F.P. caused a threefold increase in twitch tension associated with repetitive electrical responses. In Fig. 7 are shown the diphasic records of the action potentials from the eye muscle, produced by single maximal nerve volleys (*a*) before and (*b*) after an arterial injection of 100 μg. D.F.P. The muscle showed vigorous spontaneous contractions of a tension as great as those produced by maximal motor nerve volleys before D.F.P.; these contractions were also associated with repetitive action potentials (Fig. 7*c*).

The denervated muscle. In the denervated muscle D.F.P. in doses as high as 2 mg., administered directly into the artery supplying the muscle, is without any effect on the tension responses of the muscle to direct electrical stimulation (Fig. 8*b*).

A tetanic stimulation of the muscle for 5–10 sec. produces a contraction, the form of which is unchanged by D.F.P. It is followed by the typical post-tetanic potentiation, and depressant effects are absent.

Fig. 8. Tension response of cat's denervated tibialis anterior (7 days) to maximal stimulation every 10 sec. (90 V. × 1 msec.). Arterial injection of 0·5 μg. acetylcholine (*a* and *c*) and of 2 mg. D.F.P. (*b*).

The arterial injection of acetylcholine into the denervated muscle before D.F.P. produces (Fig. 8*a*) a quick initial contraction—associated with propagated action potentials—followed by a secondary development of tension which is a contracture proper (Gasser & Dale, 1926; Brown, 1937). D.F.P. has little obvious effect upon the first phase of the acetylcholine response; the second phase, on the other hand, is accentuated and prolonged, and is associated with an irreversible depression of the tension response of the muscle to direct stimulation (Fig. 8*c*).

DISCUSSION

Our results have shown that the action of D.F.P. on the mammalian nerve-muscle preparation closely resembles that of eserine. Just as the actions of eserine are explicable by its inhibition of cholinesterase, a similar mechanism appears capable of accounting for the phenomena we have observed with D.F.P. It has been found to be about five and a half times as active as an equal weight of eserine sulphate when the cholinesterase inhibiting activity of the two substances was compared on human plasma. In our present experiments, nevertheless, the quantity of D.F.P. needed to produce a potentiation of the twitch tension was several times greater than the doses of eserine found to be necessary to evoke a comparable potentiation under similar conditions (Brown, Dale & Feldberg, 1936; Bacq & Brown, 1937). D.F.P., however, has a much greater affinity for pseudo-cholinesterase than for true cholinesterase, and the latter, although virtually absent in human plasma, is the enzyme which must be inhibited if the acetylcholine released by nerve impulses is to accumulate at its site of liberation (Hawkins & Mendel, 1947).

It would appear, therefore, that the first action of D.F.P. when administered intravenously is to attach itself irreversibly to and inhibit the enormous reserve of pseudo-cholinesterase in the plasma. It is only when large doses of D.F.P. have been administered intravenously that a sufficient surplus of the inhibitor is free to attack the true cholinesterase at the neuromuscular junction. The relatively trivial effect on neuromuscular transmission of large doses of D.F.P. when given intravenously is thus in accordance with the known properties of the substance. It is probable that this factor of the preferential affinity of D.F.P. for pseudo-cholinesterase reduces the effectiveness of the drug even when it is given by the arterial route. Nevertheless, such inhibition of true cholinesterase as occurs under these circumstances, persists.

The depression of the tension response of a muscle to single nerve volleys after the initial potentiation by D.F.P. resembles, but is greater than, that produced by eserine, which was observed by several authors and discussed in detail by Brown *et al.* (1936). They pointed out that the depression was due not to a direct action of eserine but to acetylcholine persisting at the site of its liberation or locus of action when injected arterially. They further observed that the depression by acetylcholine is not confined to a curari-like action on neuromuscular transmission but extends to the whole of the muscle fibre. Our experiments provide further evidence in support of this view. During the depression which results from D.F.P. the response of a muscle is impaired, not only to incident nerve volleys, but even to electrical excitation of the muscle with currents of sufficient intensity and duration to excite its fibres directly.

In the innervated muscle, depressant effects can be produced both by tetanization of the nerve and by injection of acetylcholine. In denervated muscle, only the injection of acetylcholine produces depression after D.F.P.; tetanic stimulation of the muscle is not followed by a depression of the response. The depression produced in the innervated muscle by tetanic stimulation of its nerve must, therefore, be the result of the persistence of the acetylcholine liberated at the end-plate. Acetylcholine may act either by rendering some fibres inexcitable or by reducing the tension produced by the individual fibre, or act in both ways. It is evident that D.F.P., in the doses we have used, has by itself, no direct depressant action on muscle function.

We have observed that the depression in innervated muscle produced by acetylcholine is often persistent. The appearance, on occasion, of a secondary slow contraction in response to an arterial injection of acetylcholine suggests an explanation of the phenomenon. In denervated muscle, a sufficiently large dose of acetylcholine produces a similar but greater slow contracture, which leaves behind it a permanent derangement of the conducting system of the muscle fibre (cf. Brown, 1937). It appears not unlikely, therefore, that a similar effect of injected acetylcholine may be produced in innervated muscle after D.F.P., which allows the acetylcholine to remain in contact with the muscle fibre. The amounts of acetylcholine released by the motor impulses at the end-plate, on the other hand, probably reach the muscle fibre in too low a concentration to produce such a permanent change. The depression produced by a tetanus, therefore, is more transient and is due probably more to neuromuscular block than is the permanent depression, which follows the flooding of the whole muscle with acetylcholine when injected arterially.

SUMMARY

1. In decerebrate cats (2–3 kg.), the arterial injection of 100–200 μg. of D.F.P. causes an increase of tension response of the tibialis anterior to single maximal motor nerve volleys. This potentiation is due to repetitive contraction of the muscle fibres and is followed by a period of depressed response, during which the contraction is still repetitive. By intravenous injection, D.F.P. is effective only in very large doses (10 mg./kg.).

2. During and after the D.F.P. potentiation, stimulation of the nerve at 50–200/sec. for 5–10 sec. produces a poorly sustained contraction which is followed by a transient depression of the response to single nerve volleys; a similar but more permanent depression of the response to single nerve volleys is produced by an arterial injection of acetylcholine.

3. The depressant effects observed after D.F.P. result from an action of acetylcholine which produces neuromuscular block and a condition of inexcitability of the muscle fibre, both when the acetylcholine is injected or released at the nerve endings.

4. On the denervated muscle an arterial injection of even 2 mg. of D.F.P. has no effect. A subsequent injection of acetylcholine produces a contracture which is greater and more prolonged than that which occurs in the untreated muscle; it is followed by a profound depression of the response of the muscle to direct electrical excitation.

5. The fact that D.F.P. has a greater affinity for pseudo- than for true cholinesterase must be taken into account when the effect of arterial and intravenous injections of D.F.P. on neuromuscular transmission are compared with those of eserine.

We should like to express our thanks to Dr B. C. Saunders (Cambridge) for the supply of D.F.P.

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AN INTEGRATION OF THE TOTAL OXYGEN CONSUMPTION OF THE SHEEP FOETUS FROM THAT OF THE TISSUES

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Since the literature relating to the respiratory intensity of the mammalian embryo was reviewed by Needham (1931), Barcroft and his co-workers have investigated this problem, working with the goat and sheep, and have obtained values for the foetal oxygen uptake by measuring the oxygen tension and the blood flow through the foetal placental bed (Barcroft, Flexner & McClurkin, 1934; Barcroft, Kennedy & Mason, 1939). Their results tended to show that the values obtained earlier by Cohnstein & Zuntz (1884) were too low. More recent values for placental blood flow, by means of improved methods (Barcroft & Torrens, 1946), gave even higher figures for oxygen consumption (Barcroft & Elsden, 1946). It was felt that, although the improved experimental procedure justified reliance in the later and higher values, it was desirable to check them by an independent method since there was the possibility that experimental interference might have led to unphysiological results.

From figures obtained by Krebs (Krebs, H. A., unpublished), it seemed likely that an integration of the total oxygen consumption of the foetus from the results of tissue slice experiments might yield values comparable with those obtained by direct determination on the whole animal. The present paper describes experiments carried out in an attempt to obtain such integrated values for the sheep foetus over a wide range of ages. The results extend and, in some cases, modify those previously reported in summary form (Carlyle, 1945 *a*, *b*, *c*).

MATERIAL AND GENERAL METHODS

Foetal material was obtained from pregnant ewes of the same Welsh stock as used by Barcroft for previous investigations of this nature. The sheep were tupped at known dates. Pregnancy was interrupted by Caesarian operation under spinal anaesthesia (duracaine) as previously described (Barcroft & Mason, 1938). Where an attempt was made to estimate foetal blood volume, the foetus was killed by bleeding from the carotid arteries; in all other cases death was caused by ligation of the cord. Tissues were removed immediately after death.

Reference to Fig. 1 shows that the tissues or organs appear to fall into two main groups: those in which the dry-matter content rises throughout foetal life, and those in which it remains relatively constant until the end of gestation, when it rises rapidly. The first group contains mainly supporting structures, the second the organs. Rather surprisingly, bone shows a progressive water loss, but cartilage maintains the same content until after birth. The dry matter in each tissue varies independently, and that in the whole body is the sum of that in the parts, there being no common causal factor operating equally on all tissues.

Weights of tissues as a proportion of total body weight

Methods

The material was taken as previously described. The following tissues require special note:

Skin. The whole integument was dissected off the animal, freed from adherent subcutaneous tissue, and weighed. In foetuses older than 100 days the wool became wet owing to immersion in the saline bath when Caesarian section was performed. Before removing the skin, the wool was dried as far as possible by rubbing with dry towels. In all cases it was necessary for subsequent calculations that the weight of the actual skin as distinct from that of the wool should be known. As shaving the whole animal was impracticable, two representative areas of skin were taken, and weighed before and after shaving. Comparative figures for whole skin and shaved skin are given in Fig. 2.

Gut. The entire gut was treated as described in the preceding section, and weighed in two fractions: the stomach, including oesophagus, and intestines.

Blood was collected by bleeding out the live animal from both carotids into a weighed vessel.

Inert material. Included in this category are the wool, and the contents of the gut.

Muscle. After removal of the skin, blood, brain and viscera, the carcass was then weighed. This weight was taken to represent muscle plus skeletal structures. The muscle was then dissected away from the skeleton as cleanly as possible. This process took some time. Any part of the carcass not being worked on was kept in a damp cloth to minimize loss by evaporation. After cleaning had been completed, the skeleton was reweighed and the weight of muscle obtained by difference.

Skeleton. This presented two difficulties. It proved impossible to remove the last traces of adherent ligament and tendon by physical methods. An attempt was made to complete the cleaning by partial maceration, but this was not successful as it affected the cartilage. The figures presented for skeletal weight are thus slightly too high, but the error involved is not great. The more important problem is that of determining the relative proportions of bone and cartilage. Though in the adult animal these two constituent parts are well defined, zones of transition being small, this is not so in the foetus. Here it is difficult to speak of bone or cartilage in the sense of separate uniform tissues, the major part of the skeleton being in the process of transition, and mechanical separation impossible. But, because the skeleton forms such a large proportion of the whole organism, and since an error in the computation of its oxygen uptake would materially affect the final figure, it is necessary to know not only the relative weight of the skeleton as a whole but, also, how much is bone and how much cartilage. Here, and subsequently, we have used the term cartilage in a special sense, loosely to connote all such tissues, permeating or closely adherent to bone, having approximately the same water and calcium content and Q_{O_2} as the definitely cartilaginous structures. The procedure involved a preliminary maceration of the skeleton in water for 14 days. The resultant loss in weight is shown in Table 1 (column 1).

From the appearance of the macerated skeleton it was apparent that not all the cartilage had been removed. To determine the amount of cartilage unmacerated, each skeleton, after maceration, was divided into three parts: (*a*) femur (=bone); (*b*) suprascapular cartilage (=cartilage); and (*c*) the rest of the skeleton. Each of these was estimated for dry-matter content and for calcium.

From these results the ratio of bone to cartilage in the macerated skeleton was calculated. Combination of these results gives an estimate of the cartilage and bone in the fresh skeleton (Table 1, columns 4 and 5).

In two animals (51-day foetus and 21 yr. old ewe), a simpler procedure was adopted. A dry-weight determination was carried out on the fresh skeleton by means of three samples as before, and the ratio in the fresh skeleton calculated directly. These results are incorporated in Table 1, where the average dry weights for the whole skeleton are also given (column 6).

TABLE 1. The proportion of bone and cartilage in the fresh and macerated skeleton (expressed as percentage of total body weight)

	Loss on maceration (cartilage) 1	Macerated skeleton		Fresh skeleton		
		Cartilage 2	Bone 3	Cartilage 4	Bone 5	Dry weight % 6
Foetus 51 days	—	—	—	5·9	5·8	25
109 ,,	18·4	3·3	4·3	22	4·3	28
112 ,,	15·3	4·9	5·8	19	5·8	29
136 ,,	12·9	2·8	7·4	16	7·4	28
143 ,,	10·3	5·8	6·9	16	7·0	28
Lamb 6 days	8·8	6·1	10·1	15	10	36
Adult 2 yr.	3·9	1·7	5·0	5·6	5·0	49
Adult 21 yr.	—	—	—	4·4	9·1	67

Results

The results are given in Fig. 2. Owing to the small number of animals represented by each value, there is a certain irregularity due to individual variation. In spite of this, two points stand out clearly: the different organs do not all develop at the same rate; while some are growing faster than

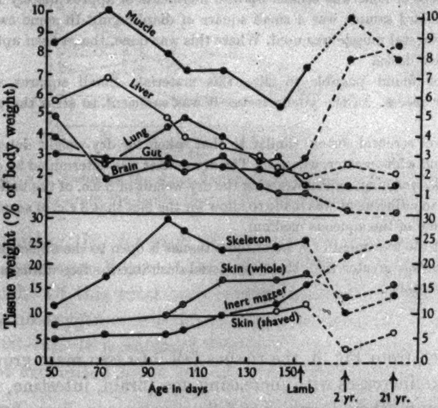

Fig. 2. The weight of tissues of the sheep foetus, lamb and adult sheep, as a percentage of the total body weight. Muscle is plotted at ¼ scale along the ordinate.

the animal as a whole, e.g. skeleton and skin, others are growing more slowly, e.g. brain, muscle and gut. As in the previous section, there appears to be considerable tissue independence.

Oxygen uptake of the tissues

Methods

The oxygen consumption was measured in Warburg manometers by the simplest method, i.e. oxygen uptake in glucose-phosphate-buffer with pure oxygen as the gas phase, carbon dioxide being absorbed with caustic potash. The preparation of the different tissues is described below.

Owing to the limited number of animals available, it was desirable that, if possible, values for all the tissues studied should be obtained from each animal and, as only six experimental manometers could be utilized at any one time, only three of the tissues could be studied in the normal state, that is immediately after death of the animal, and the respiration of the others was examined after a lapse of anything up to 2 hr. Fuhrman & Field (1943) had reported that certain tissues in the rat could be kept at 2° C. for an hour without alteration in their oxygen uptake as subsequently measured at 37° C. Preliminary experiments with this technique for foetal tissues of the sheep indicated that, with the exception of brain, the oxygen consumption after such treatment was not significantly different from that of the fresh tissue. Accordingly, for oxygen uptake of brain, liver and blood, material from the freshly killed animals was used. Other material was taken at the same time and kept in Ringer phosphate at 2° C. until required. Throughout the experiment, the second series of determinations was made after 1 hr. on muscle, stomach and intestine, and the third series on lung, cartilage and bone after 2 hr. After temperature equilibration of the flasks, readings were taken at 15 min. intervals, duplicate samples of each tissue being used. From liver, lung, cartilage and skin, tissue slices were cut in the ordinary way. Treatment of the other tissues was as follows.

Bone. A small piece of bone was broken up into fragments of approximately 1 mm. cube.

Muscle. The standard sample was a small square of diaphragm. In some cases a third flask containing teased skeletal muscle was used. Where this was done, the oxygen uptake agreed with that of the other two flasks.

Gut. It was not found possible to slice this material. Small squares were taken and teased into smaller pieces. In the young foetus it was sufficient to strip the mucosa from the muscularis.

Brain. A sample of cerebral cortex, similar to that taken for dry-weight determinations, containing both grey and white matter, was used. The dry matter was determined by drying the whole contents of the flask, weighing, and subtracting the dry weight of 3 c.c. of the buffer as determined separately. Later an adjustment was made to allow for the fact that 3 c.c. of suspension contained less than that volume of the aqueous medium.

It is realized that the treatment of some of these tissues is open to the objection that the thickness of the particles was greater than that considered desirable for free diffusion of oxygen. We regard this as unavoidable.

Results

As will be seen from Fig. 3, the tissues fall into two main groups: those in which O_2 uptake increases with increasing age (brain, intestine, stomach and possibly lung), and others in which it falls.

It would be interesting to speculate as to the significance of the sharp peak at birth shown by the curve for brain. Also interesting is the fact that the values for the 21 yr. old sheep show no general fall, although this animal appeared senile.

Integration of the total oxygen uptake

From the results described above it is possible to estimate the oxygen consumption for the whole animal from the uptake of the isolated tissues. Total oxygen consumption is expressed as c.c. O_2/kg. body weight/min. From the percentage dry matter of the tissues or organs and their percentage of the total body weight, the dry matter of each in g. can be calculated for each kg. of body weight. The expected oxygen uptake of that weight of dry matter can then be obtained. By doing this for each of the selected tissues, a certain oxygen uptake, x c.c., corresponding to tissues which constitute y% of the total body weight is found. Of this total body weight a certain fraction, z%, is inert matter having no oxygen consumption. Since we have only dealt with certain tissues, $y<(100-z)$. It is assumed that the average oxygen consumption of the tissues not accounted for is not materially different from the average of those measured, and, accordingly, that the figure given by $\frac{x\,(100-z)}{y}$ will approximate to the oxygen uptake of the whole. It will be seen from the tables that the selected tissues constitute, in all cases, not less than 88% of the total body weight. Details of these calculations at one selected age are given in Table 2. The final figures are summarized in Table 3.

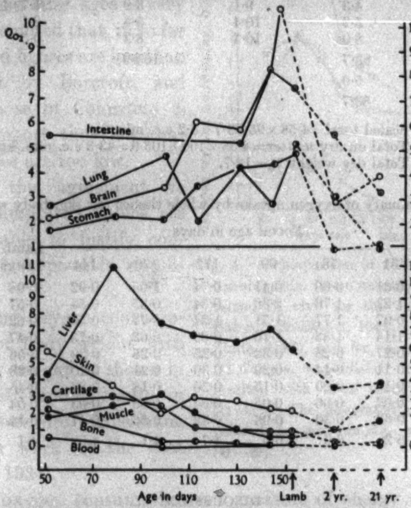

Fig. 3. Q_{O_2}'s of tissues of the sheep foetus, lamb and adult sheep. The value for brain in the lamb (13·6) is not shown in the figure.

Table 2. Detailed calculation for integrated oxygen uptake of whole foetus at 51 days

Tissue	Body weight (%)	Dry matter (D.M.) (%)	g. D.M./kg. body weight	Q_{O_2}	Oxygen uptake c.c./min./kg. body weight
Skin	7·8	8·2	6·4	5·7	0·61
Cartilage	5·9	21·7	12·8	2·8	0·60
Bone	5·8	29·4	17·0	2·2	0·62
Muscle	35·3	9·1	32·2	1·8	0·97
Liver	8·2	19·4	15·9	4·3	1·14
Intestine	1·6	12·6	2·0	5·5	0·18
Stomach	1·6	12·6	2·0	2·6	0·09
Lung	4·3	9·1	3·9	1·7	0·11
Brain	5·2	10·4	5·4	2·1	0·19
Blood	8·0	10·4	8·3	0·5	0·07
	83·7		105·9		4·58
Dead matter	5·0				
Total	88·7				

Scaled total = 4·58 × 95/83·7 = 5·2 c.c./min./kg.
Total on dry matter = 4·58 × 1000/105·9 = 43·3 c.c./min./kg.
Total dry weight % = 12·7.

Table 3. Summary of oxygen uptake by whole tissues (c.c./kg. body weight/min.)

	Foetal age in days						Lamb	Adult	
Tissue	51	78	99	112	130	144	6 days	2 yr.	21 yr.
Skin	0·61	0·60	0·41	0·74	1·05	0·92	0·98	0·18	0·87
Skeleton	1·22	1·76	2·36	0·54	0·35	0·54	0·57	0·10	0
Muscle	0·97	1·77	1·71	1·34	0·77	0·50	0·62	2·1	2·0
Liver	1·14	1·83	1·16	0·83	0·63	0·73	0·47	0·47	0·33
Gut	0·27	0·28	0·32	0·25	0·28	0·39	0·56	1·0	0·65
Lung	0·11	0·14	0·29	0·30	0·21	0·20	0·28	0·16	0·05
Brain	0·19	0·09	0·15	0·30	0·18	0·26	0·45	0·04	0·004
Blood	0·07	0·06	0·05	0·04	0·05	0·03	0·04	0·05	0·09
Other tissues	0·62	0·30	0·06	0·32	0·56	0·47	0·14	0·50	0·19
Total	5·2	6·9	6·5	4·7	4·1	4·0	4·1	4·6	4·2

DISCUSSION

It is necessary now to consider the validity of the final integrated figures, and to compare them with values obtainable for the actual oxygen uptake of the whole animal. Differences might be found on both technical and physiological grounds. The indirectness of the method favours the introduction of additive errors at each stage, producing an error of large magnitude in the final figure. A particularly large error might occur in the determination of oxygen uptake, owing to the difficulties of handling some of the tissues. Furthermore, in respect to oxygen uptake, the conditions under which the values were obtained differed in important respects from those present in the intact animal. These include the difference in partial pressure of oxygen and carbon dioxide, the absence of bicarbonate from the medium, and, in some cases, mechanical disturbance of the tissue. At best, there is reason to suppose that the integrated figures would be somewhat lower than those obtaining in vivo since the tissues would be under basal conditions; in particular we have to consider the effect of loss of voluntary muscle tone, cessation of such bodily activities as the heart-beat and the changed conditions of secretory activities.

In view of these a priori arguments, the agreement shown, when the figures are compared with those obtained on the whole animal, is remarkable. It will be seen that although the figures obtained in vivo are substantially higher at the earlier ages, they are of the same order of magnitude, and show the same variation with respect to age. Agreement at the later ages is very close. It may be noted that, in so far as the integrated figures are less than those obtained by Barcroft, and higher than those of Cohnstein & Zuntz, they support the view that the earlier figures are too low.

In view of this agreement we feel that the integrated values are sufficiently reliable to justify consideration of their possible significance.

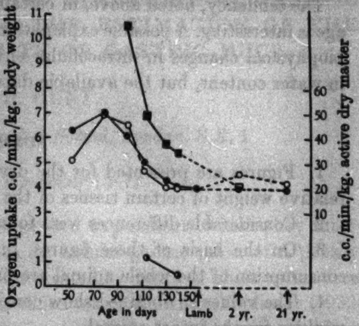

Fig. 4. A comparison of values of total oxygen consumption, in c.c./min./kg. body weight, obtained in vivo for the sheep foetus (■—■ Barcroft & Elsden, 1946; ◑—◑ Cohnstein & Zuntz, 1884), and for the resting adult sheep (□ Carlyle, unpublished indirect calorimetry results), with those obtained by integration (○—○). The integrated values are also expressed as c.c./min./kg. active dry matter (●—●).

The decreasing oxygen consumption per unit of body weight with increasing body weight as shown in Fig. 4 agrees with similar results in the earlier literature (Needham, 1931) and later work for the goat (Barcroft, *et al.* 1934).

In so far as oxygen consumption is a measure of energy utilization, this indicates a falling metabolic rate with increasing body weight, as is implied in the formulae widely used for predicting metabolic rates in adult animals from the body weight, such as that put forward by Brody, Procter & Ashworth (1934):

$$Q=70\cdot5\times M^{0\cdot734},$$

where Q=cal./day, and M is the body weight in kg. The integrated figures for oxygen consumption obtained here suggest that this correlation holds for the sheep foetus. We have examined the data presented to see if they elucidate this relationship. They indicate that four changes are likely to affect the total oxygen consumption per unit of fresh weight: (1) progressive loss of tissue water, (2) increase of inert material, (3) change in body composition in favour of tissues of low oxygen uptake, (4) tendency in some tissues at least towards a lowered oxygen utilization rate per unit of dry matter.

Comparison of the curves for oxygen uptake per unit of total body weight, and per unit of active dry matter (Fig. 4), suggests that the change in total uptake is not determined by the simple operation of the first two factors, but is to be explained by the change in body composition in favour of tissues of a relatively lower Q_{O_2}, together with the decrease in the Q_{O_2} of certain tissues.

The tendency, noted above, in certain tissues for Q_{O_2} to fall with increasing age is interesting. A possible explanation would be that it was consequent upon biophysical changes in intracellular environment resulting from the decrease in water content, but the available data tend to disprove such a contention.

SUMMARY

1. Figures are presented for the dry-matter content, oxygen uptake, and relative weight of certain tissues of the sheep foetus, lamb and adult ewe.

2. Considerable differences were found between the tissues in each series.

3. On the basis of these figures, integrated values for the total oxygen consumption of the whole animal are presented.

4. The values so obtained show considerable agreement with those of other workers for the intact animal.

5. The validity of these results and their possible significance are discussed.

The author wishes to acknowledge his very great indebtedness to the late Sir Joseph Barcroft, under whose direction the work was carried out, for his advice and interest throughout these experiments.

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THE PRESENCE OF A PEPTIC SYNERGIST IN GASTRIC JUICE: ITS IMPORTANCE IN THE ESTIMATION OF THE PROTEOLYTIC ACTIVITY OF GASTRIC JUICE

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In 1945, Bucher, Grossman & Ivy described experiments which they interpreted as demonstrating the presence, in human gastric juice, of a pepsin-inhibitor/pepsin complex, which was dissociated by dilution. Their estimations of peptic activity were made with a modification of the Anson & Mirsky (1932) method. This method uses the increase in phenolic hydroxyl groups soluble in trichloroacetic acid and reacting with Folin & Ciocalteu's (1927) phenol reagent, as a quantitative index of the digestion of a substrate of carboxyhaemoglobin. As a result of their experiments they suggested that in estimating the peptic activity of human gastric juice it was necessary first to dilute the juice a hundredfold in order to eliminate the effect of the inhibitor. This point was obviously of some significance since it invalidated all estimations of the peptic activity of gastric juice in which this requirement had not been satisfied.

This paper describes unsuccessful attempts to confirm their work and provides a possible explanation of the discrepancy by the demonstration of a peptic synergist in gastric juice.

METHODS

The proteolytic activity was estimated by a modification of the Anson & Mirsky method (Hunt, 1948) in which the carboxyhaemoglobin substrate is replaced by plasma proteins.

It appeared from *a priori* consideration that if the product of the concentration of the gastric juice and the time of digestion were kept constant, in the absence of an inhibitor, the degree of colour developed, corresponding to phenolic hydroxyl groups, would be constant. On the other hand, if an inhibitor were present, a diluted sample with a correspondingly increased digestion time should be more active than an undiluted sample, owing to the dissociation of the inhibitor-pepsin complex.

RESULTS

Twelve fresh 'resting juices' and seven fresh post-prandial juices were obtained from students. Each juice, diluted with an equal quantity of a solution of HCl (pH 2·1), was allowed to act on the substrate for 10 min. at

35° C. and the colour corresponding to the increase in reactive phenolic hydroxyl groups was determined with Folin & Ciocalteu's reagent in a photoelectric colorimeter. It had previously been shown that the buffer capacity of the substrate was such as to make the estimation of the proteolytic activity of the gastric juice independent of its acidity, provided its pH lay between 1·6 and 4·1 (Hunt, 1948). Samples of the same gastric juices, diluted 1 in 20 with the same solution of HCl, were then allowed to act on the substrate for 100 min. and the colour was similarly measured. A total of 54 pairs of such determinations was made. The mean difference between the colour produced at the high dilution and low dilution was +0·758%, standard error of the mean ±1·034%. The findings were the same with substrates of plasma, serum or boiled serum. These results appeared to provide a good reason for believing that the diluted juice did not in fact have any enhanced activity.

Bucher *et al.* reported that samples of commercial and pure pepsin did not show the removal of an inhibiting effect when diluted. In this study similar results have been obtained for samples of crude commercial pepsin and for a highly purified specimen which gave a U.S.P. value of 1 in 45,000.

In calculating their results, Bucher *et al.* used a standard curve relating proteolytic activity to colour developed in the reaction. This curve was produced experimentally from solutions of pepsin, not gastric juice, so that their results really expressed the 'pepsin-equivalents' of the samples examined.

The proteolytic activity of gastric juice may only be legitimately expressed in 'pepsin-equivalents' if the curves for pepsin solution and gastric juice are identical. Therefore curves relating proteolytic activity to the colour difference between control and test were prepared for gastric juice and for commercial pepsin. These curves are shown in Fig. 1. They have been superimposed at *A*, thus arbitrarily making a solution of commercial pepsin (1·5 g./100 ml.) equivalent to a concentration of 158 pepsin units (Hunt, 1948). It may be seen that the curves are not identical. Further, the discrepancy is such as to produce an error in reading the proteolytic activity of gastric juice from the curve for pepsin which might be interpreted as the removal of an inhibitor with dilution. For example, a gastric juice having an activity of 158 pepsin units/ml. would, after dilution with an equal volume of a solution of HCl, give an optical density of 0·64 on estimation. If this colour is used to read the activity of the gastric juice from the curve for pepsin, instead of the curve for gastric juice, it corresponds to 89 pepsin units instead of the expected value of half 158, namely 79, i.e. 50% dilutions would appear to remove inhibitor to the equivalent of 10 units.

Bucher *et al.* reported that the amount of inhibition varied from one gastric juice to another; it is therefore relevant to inquire if the curve given in Fig. 1 for gastric juice differs significantly from that for commercial pepsin and whether the curve is sufficiently constant from one juice to another to allow it to be used as a standard. The curve given in Fig. 1 for gastric juice was the mean curve for 138 points obtained from ten 'resting juices' and two obtained after injection of histamine. The constancy of this curve was tested with eight different samples of human gastric juice. The colours developed at various dilutions were determined, and the equivalent proteolytic activity was obtained from the standard curve for gastric juice. This directly obtained value

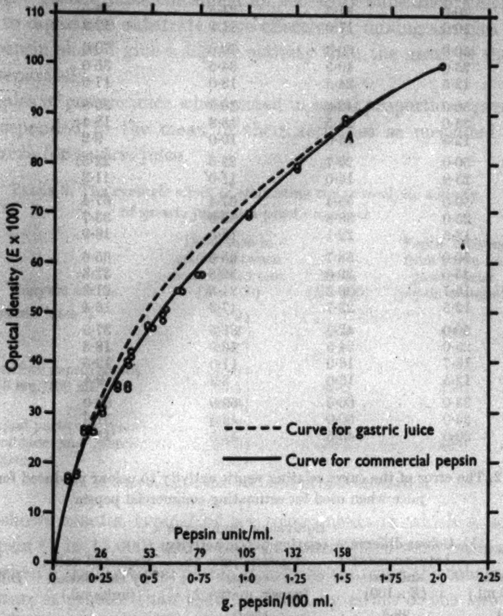

Fig. 1. Curves relating the proteolytic activity of gastric juice and commercial pepsin to the colour difference between control and test.

was then compared with the theoretical activity calculated from the known degree of dilution and the activity of the original juice. The results of twenty-seven duplicate estimations are shown in Table 1. The mean percentage difference between the two sets of readings is −1·178 whilst the standard error of a single difference is 3·15%. Since these experiments involved estimations at very low concentrations of gastric juice, without increasing the incubation time as would be done in practice, the agreement may be considered satisfactory.

Table 1. Variation of the relationship between peptic activity of gastric juice and the difference in colour between the control and test with different gastric juices

Subject	Gastric juice conc. (%)	Colour difference between control and test ($E \times 100$)	Peptic activity (units/ml.)	Peptic activity calculated from value obtained at 50% dilution (units/ml.)	Difference (%)
A	50·0	56·8	61·2	61·2	—
	40·0	49·5	47·5	48·9	− 2·8
	30·0	44·0	39·4	36·7	+ 6·5
	20·0	31·2	24·5	24·5	0
	10·0	17·0	11·9	12·2	− 2·5
B	50·0	60·3	70·0	70·0	—
	25·0	40·5	34·5	35·0	− 1·4
	12·5	24·5	18·0	17·5	+ 2·8
C	50·0	42·0	36·8	36·8	—
	25·0	25·5	18·8	18·4	+ 2·2
	12·5	15·0	10·0	9·2	+ 8·0
D	50·0	29·7	22·5	22·5	—
	25·0	16·0	11·0	11·3	− 2·6
E_1	50·0	59·4	67·4	67·4	—
	25·0	38·8	32·6	33·7	− 3·3
	12·5	22·1	16·0	16·9	− 5·2
E_2	50·0	58·7	65·6	65·6	—
	25·0	39·0	32·8	32·8	0
	16·7	28·3	21·5	21·9	− 1·8
	12·5	23·7	17·3	16·4	+ 5·4
F	50·0	42·5	37·5	37·5	—
	25·0	24·5	18·0	18·8	− 4·3
	16·7	16·0	11·0	12·5	−12·0
	12·5	13·0	8·9	9·4	− 5·3
G	33·0	60·0	69·0	69·0	—
	25·0	50·0	48·5	51·8	− 6·3
	20·0	45·5	41·5	41·4	+ 0·2

Table 2. The error of the curve relating peptic activity to colour produced for gastric juice when used for estimating commercial pepsin

Pepsin (g./100 ml.)	Colour difference between control and test ($E \times 100$)	Equivalent from curve relating peptic activity of gastric juice to colour (pepsin units/ml.)	Expected value by calculation (units/ml.)	Difference (%)
1·50	88·5	158·0	—	—
1·00	69·7	95·0	105·3	− 9·8
0·75	58·0	64·2	79·0	−18·7
0·625	54·0	54·0	65·8	−17·9
0·500	46·5	42·8	52·7	−18·8
0·250	30·4	23·5	26·3	−10·6
0·125	18·4	12·9	13·2	− 2·3

If the values obtained with the commercial pepsin are similarly examined it may be seen in Table 2 that at all concentrations above 0·25 g. pepsin/100 ml. there is a considerable difference between observed and calculated values. Since the differences are at least three times the mean standard error of the curve for gastric juice as assessed from Table 1, it appears likely that the curves for gastric juice and commercial pepsin are different. Lest the discrepancy between the curves should be considered as due to a chance variation in substrate or commercial pepsin, a few additional points have been obtained for another commercial pepsin with a second sample of substrate. All the experimental points obtained for commercial pepsin have been included in Fig. 1.

Preliminary experiments have been made to investigate this difference between the course of proteolysis with human gastric juice and commercial pepsin. If the gastric juice contained an accessory substance which assisted the pepsin to digest the substrate more effectively, mixing samples of gastric juice and pepsin should give a higher activity than the mean of the samples estimated separately.

Two samples of gastric juice when mixed in equal proportions gave a colour which corresponded to the mean of their activities as measured from the standard curve for gastric juice.

Table 3. The synergic effect of estimating the proteolytic activity of gastric juice and pepsin together

Enzyme source	Difference in colour between control and test ($E \times 100$)	Mean ($E \times 100$)	Peptic activity from curve for gastric juice (pepsin units/ml.)
Gastric juice A	44·9 44·7 45·1	44·9	40·5
Purified pepsin about 15 mg./100 ml.	40·0 40·5 41·0	40·5	35·0
Equal parts of pepsin solution and gastric juice	50·2 50·2 50·2	50·2	49·0

1 ml. of enzyme solution was used in each estimation.

Table 3 shows results typical of six experiments in which a solution of purified pepsin (1 in 45,000) and a human gastric juice were estimated separately and when mixed in equal parts. It may be seen that the colour produced by the mixture is greater than that produced by either of the two solutions separately. If the curve relating activity to colour were the same for pepsin and gastric juice the expected optical density produced by the mixture would be about 0·427. The difference between this and the colour actually obtained corresponds to an optical density of 0·075, which is equivalent to 10 pepsin units on this part of the curve. Since the standard error of an individual estimation by the method employed is 2·6% (Hunt, 1948) the difference is six times the standard error of a triplicate estimation, so that there can be no doubt of its significance. This enhancing effect of gastric juice upon pepsin was not shown when gastric juice or a solution of purified pepsin, previously heated to 80° C. for 20 min. and cooled, was added to a solution of pepsin. The heated solution of pepsin when containing only about 15 mg./100 ml. had a marked inhibiting action both on gastric juice and pepsin. These results are compatible with the presence of a synergic enzyme in the gastric juice but do not, however, exclude other possibilities.

DISCUSSION

The difference between the curves relating proteolytic activity to the colour developed for commercial pepsin and gastric juice provides a reasonable explanation of the failure to demonstrate the inhibiting complex postulated by Bucher *et al.* There are, however, other possibilities. It might be suggested that plasma proteins are a special substrate upon which the effect of diluting gastric juice is not apparent. If this is the case, it is an advantage in the use of this substrate, but one which, according to the results of Ihre (1938), it shares with casein. It must be admitted that although the difference between the two curves accounts qualitatively for the effect described by Bucher *et al.*, it does not account for the twofold enhancement described by these workers. It seems possible, therefore, that the two curves may be even less coincident with the more dilute substrate of carboxyhaemoglobin employed in their work.

The results of the estimation of mixtures of pepsin and gastric juice are compatible with the hypothesis that the observed enhancement is due to a second enzyme in gastric juice which converts the substrate into a form more readily digested by pepsin.

When pepsin and gastric juice are mixed, the added pepsin acts on an altered substrate and shows enhanced activity. Since Northrop (1939) has already shown the presence of gelatinase in gastric juice, there is nothing improbable in this suggestion. When the first experiments were made to establish the relationship between the proteolytic activity of gastric juice and the difference in colour between the control and test, a stored sample of gastric juice was used. Subsequent comparison with curves made from fresh samples showed that the stored juice gave an increased rate of digestion at low concentrations as compared with fresh juice. This observation may perhaps be coupled with the well-known finding that pepsin solutions are unstable whilst gastric juice shows remarkable stability in its proteolytic powers. This relative stability of proteolytic activity of gastric juice might be due to a gradually increasing proportion of the substrate made available to a second more active enzyme as the pepsin became progressively inactivated. The hypothesis that the difference observed between gastric juice and commercial pepsin is due to a synergic enzyme receives support from the work of Kraut & Tria (1937) who, using a method originally described by Brücke in 1875, prepared a proteolytic enzyme from gastric mucosa which was quite distinct from pepsin as prepared by Northrop. Their enzyme acted synergically with pepsin and had an initial reaction rate greater than pepsin, two properties which have been demonstrated for gastric juice. It appears, therefore, that their enzyme may be present in gastric juice as well as in gastric mucosa.

As a consequence of the difference between the curves for commercial pepsin and gastric juice, it appears that the relationship between the degree of proteolytic activity and the index substance measured in any method of estimating the proteolytic activity of gastric juice, should be established with gastric juice and not with pepsin. The proteolytic activity of gastric juice may only legitimately be expressed in terms of weights of pure pepsin if the concentration of a standard solution is defined.

SUMMARY

1. Using a modification of the Anson & Mirsky method of estimating proteolytic activity, no evidence of a pepsin-inhibitor/pepsin complex dissociating on dilution has been found.

2. An hypothesis, supported by experiment, is put forward to account for the positive findings of previous workers.

3. The reproducibility of the curve relating peptic activity of gastric juice to the concentration of the products of digestion is delimited for eight gastric juices.

4. The difference between this relationship for commercial pepsin and human gastric juice is shown.

5. Preliminary experiments have given results compatible with the presence of synergic enzyme in gastric juice.

6. The significance of this finding as it relates to methods of estimating the proteolytic activity of gastric juice is discussed.

I am indebted to Messrs Parke Davis & Company for a supply of purified pepsin.

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ACETYLCHOLINE SYNTHESIS IN DIFFERENT REGIONS OF THE CENTRAL NERVOUS SYSTEM

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In the present paper an attempt has been made to map out, in the central nervous system of the dog, the distribution of the enzyme (or enzyme system) which synthesizes acetylcholine. It has been shown that this enzyme can be extracted from acetone-dried tissue (Feldberg & Mann, 1944) and that such extracts, when incubated aerobically, form large amounts of acetylcholine in the presence of KCl, $MgCl_2$, choline, cysteine, citrate and adenosine-triphosphate (Nachmansohn & Machado, 1943; Nachmansohn, John & Waelsch, 1943; Nachmansohn & John, 1945; Feldberg & Mann, 1944, 1945, 1946; Feldberg & Hebb, 1947). Since this method is applicable to very small amounts of tissue, it was possible to compare the enzyme concentration in various quite small regions of the central nervous system.

METHODS

Dissection. The dogs were anaesthetized with ether, and bled. The brain and spinal cord were taken out as rapidly as possible. The nervous tissue was divided into several pieces and parts not immediately wanted were kept in the refrigerator whilst the dissection of the first samples was carried out. The excised samples were at once dried with cold acetone. The number of regions tested in a single experiment was limited, since care was taken that the time interval between excision of the first and the last sample did not exceed 2 hr. Samples kept in the cold for such a period did not lose much activity, certainly not more than 20%. In addition there was satisfactory agreement in control experiments in which the order of preparing the samples from different regions was varied. Samples from the tracts and horns of the spinal cord and from the region of the supraoptic nuclei were cut out with a small knife after freezing the tissue. Such freezing for a short time did not affect the ability of the tissue to synthesize acetylcholine.

Details about the dissection of those regions which are macroscopically ill-defined are as follows. Samples from the cerebral cortex and cerebellar cortex were obtained by cutting off the grey matter from the underlying fibres with fine scissors. The cortical areas were identified with the help of the maps published by Klempin (1921). The region called 'cerebellar nuclei' is that part

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of the cerebellar medullary body which contains the nuclear masses but it also contains a large number of fibres. The region of the nuclei gracilis and cuneatus contains many fibres from the corresponding tracts. The position of most other nuclei (nucleus vestibularis, motor nucleus of the vagus with hypoglossal nucleus, nucleus ruber and substantia nigra) can be identified macroscopically, but their cells are scattered between fibres of varied origin. There is no sharp definition of the different parts into which the internal capsule was divided; these were obtained by sectioning the brain horizontally and taking in succession fibres from an anterior, a posterior and an intermediate region. The optic tracts were, at first, used as a whole. Later on they were split longitudinally in such a way that one-half contained the latero-ventral and the other half the medio-dorsal fibres. A region comprising the supraoptic nucleus was obtained in the following way. A block from each hemisphere, containing half the chiasma and one optic tract, was frozen hard; then a horseshoe-shaped band of tissue, a few millimetres in thickness and width, and immediately adjacent to the medial part of the optic tract, was cut out of each block.

Assay of enzymatic activity. The method was essentially the same as that described by Feldberg & Mann (1945, 1946). The acetone-dried tissue was extracted with 0·9% NaCl but the extracts were incubated without first centrifuging off the insoluble material as was done in the experiments of Feldberg & Mann. Special care was taken that the different samples contained the same proportion of dried tissue and of saline solution. This is necessary since the rate of synthesis of acetylcholine in brain extracts is dependent on the concentration of some heat stable tissue constituent called the 'activator' (Feldberg & Mann, 1945, 1946) or the 'coenzyme of acetylation' (Lipmann, 1946). Thus, if different amounts of a particular tissue were suspended in the same volume of fluid, the concentration of the activator would vary and the rate of synthesis per g. tissue change accordingly.

There is the possibility, however, that the concentration of activator varies in different parts of the nervous system. This would introduce a source of error in our estimations, but the error would be small, because, in the presence of adenosinetriphosphate and citrate, the addition of activator, even in optimal concentration, produces a relatively small increase in the rate of synthesis. Furthermore, from the results obtained by Feldberg & Mann (1946) it appears that, if there are any variations in the concentration of activator in different parts of the nervous system, they are too small to interfere with the quantitative aspect of our assay.

When sufficient dried tissue was available, 50 mg. were ground in 4·5 c.c. of 0·9% NaCl solution to which had been added 6 mg. KCl, 2 mg. $MgCl_2$, 2 mg. NaF, 4·5 mg. cysteine hydrochloride, 0·5 mg. eserine sulphate, 3 mg. choline chloride, 12 mg. sodium citrate and 0·4 mg. adenosinetriphosphate-pyro P. When less than 50 mg. but more than 8 mg. dried tissue were available, an aliquot part of the incubation fluid was used. With less than 8 mg. dried tissue, the volume of fluid would have become too small for the assay to be practicable. Therefore the following modification was introduced. A solution of inactivated brain powder was prepared by boiling 100 mg. acetone-dried brain tissue in 4 c.c. 0·9% NaCl solution. The sample was then set up so as to contain an extract of 25 mg. of dried tissue of which 7 mg. or less were contributed by the tissue under investigation and the remainder by the inactivated brain powder. The sample was then dealt with like any other sample of 25 mg. dried tissue. In addition, a separate blank sample was incubated which contained the extract of 25 mg. inactivated brain powder and the usual constituents of the incubation mixture.

After incubation for 1 hr. at 37° C., all samples were acidified with HCl, boiled for 1 min. and stored in the refrigerator. Before assay, they were neutralized and then tested for acetylcholine on the eserinized rectus muscle of the frog with the following precautions. The larger samples were assayed against acetylcholine made up in the same volume of the test solution, from which the acetylcholine had previously been removed by short treatment with hot alkali. The smaller samples, to which extract of inactivated brain powder had been added, were assayed against acetylcholine made up in the same volume of the incubated blank sample. This was done since the test solution contained so little enzyme, and therefore so little acetylcholine, that no material could be spared to prepare a blank by boiling part of it with alkali.

RESULTS

Great individual variations were found in the synthesizing power of different brains, but the ratio between the synthesis in different regions varied little from brain to brain. For this reason, only the synthesizing power of the thalamus, which was examined in each experiment, is stated in Table 1 in absolute figures, i.e. in μg. acetylcholine formed in 1 hr. by 1 g. dried tissue, whereas the values for all other regions are expressed as percentages of this figure. The last column in Table 1 is the mean value of these percentages.

In no region of the central nervous system was the ability to form acetylcholine as high as it is in the anterior roots. This may be due to the fact that hardly anywhere in the C.N.S. do we come across a region consisting of a single kind of fibre only or of cells which are not lying between fibres of varied origin. The highest figures for the central nervous system were obtained from those regions where the spinal and cranial motor nerves originate, i.e. the anterior horns and the hypoglossal and vagal (motor) nuclei. These regions have about 70% of the activity of the anterior roots. At the lower end of the scale we find, first, the sensory nerves and tracts (optic and acoustic nerves, funiculi gracilis and cuneatus); secondly, the pyramidal fibres (both in the cord and in the cerebral peduncles); thirdly, the cerebellar cortex. The activity found in these regions is hardly greater than that of many non-nervous tissues like heart ventricle, skeletal muscle, spleen and liver.

Unlike the cerebellar cortex, the cerebellar peduncles have a moderate synthetic power; this is higher for the upper and middle than for the lower peduncle which carries the connexions with the cord. The activity of the region of the cerebellar nuclei is intermediate between that of cortex and peduncles.

The enzymatic activity in the white matter of the corpus callosum and of the internal capsule is a little higher than that of the pyramidal tracts. In the internal capsule, the most caudally situated fibres contain the smallest quantity of enzyme.

Of the remaining values listed in Table 1, the highest are those for the caudate nucleus, the region of the supraoptic nuclei and the cornu ammonis. The rate of synthesis of acetylcholine in these parts is usually somewhat greater than in the thalamus. In the cerebral cortex the enzyme content is always smaller. It is comparatively uniform even in areas functionally and architecturally as different as area 4 (motor), area 3 (somaesthetic), area 17 (visual) and area 51 (olfactory). The differences between the areas, however, are consistent in spite of their small size. It is noteworthy that the highest activity does not reside in the motor but in the olfactory area 51, particularly since another olfactory centre, the cornu ammonis, was found among the more active parts of the brain.

TABLE 1. Synthesis of acetylcholine in different regions of the nervous system

Exp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Thalamus (µg. acetylcholine/g. powder)	*270*	*400*	*370*	*220*	*250*	*260*	*260*	*210*	*230*	*250*	*280*	*520*	*520*	*300*	*500*	
	Synthesis expressed as percentage of value for thalamus															Mean
Area 4	89	60	—	68	68	81	62	69	—	68	—	—	—	—	—	71
Area 3	59	55	—	—	—	—	—	—	—	—	—	—	—	—	—	57
Area 17	56	45	—	59	72	54	62	60	59	—	—	—	—	—	—	58
Area 51	—	—	—	—	—	—	—	86	74	84	—	—	—	—	—	81
Cerebellar cortex	7·4	8·7	6·7	11·4	—	—	—	—	—	—	—	—	—	—	—	8·5
Cerebellar nuclei	29	20	13·5	—	—	—	—	—	—	—	—	—	—	—	—	21
Upper cerebellar peduncle	41	34	73	73	48	69	77	—	—	—	—	—	—	—	—	59
Middle cerebellar peduncle	—	—	—	45	60	52	85	—	—	—	—	—	—	—	—	60
Lower cerebellar peduncle	—	—	—	45	28	20	52	—	—	—	—	—	—	—	—	36
Caudate nucleus	115	155	149	123	92	—	—	—	—	—	—	—	—	—	126	127
Red nucleus	—	—	—	—	—	—	—	152	83	44	61	—	—	97	—	87
Substantia nigra	—	—	—	—	—	—	—	67	57	48	52	—	—	32	—	51
Lateral geniculate body	107	87	111	115	—	—	—	—	—	—	—	—	—	—	—	105
Superior colliculus	96	97	103	118	—	—	—	—	—	—	—	—	—	—	—	104
Medial geniculate body	70	46	65	—	—	—	—	—	—	—	—	—	—	—	—	60
Inferior colliculus	56	27	49	—	—	—	—	—	—	—	—	—	—	—	—	44
Bulbus olfactorius	52	30	—	36	—	50	73	86	—	—	—	—	—	—	—	55
Cornu ammonis	—	—	—	—	—	—	—	148	74	112	97	—	—	—	—	108
Corpora mamillaria	—	—	—	—	—	—	—	—	44	—	54	—	—	40	—	46
Infundibulum	11	22	—	—	—	—	—	48	—	—	—	—	—	—	—	27
Nucleus supraopticus	—	—	—	—	—	—	104	—	130	112	—	—	—	—	—	115
Anterior horns	—	—	—	—	—	169	123	167	—	—	—	—	—	—	—	153
Hypoglossal and motor vagal nuclei	—	—	—	—	—	150	181	286	96	176	—	—	—	—	—	178
Posterior horns	—	—	—	—	—	92	92	124	—	—	—	—	—	—	—	103
Nuclei gracilis and cuneatus	—	—	—	—	—	85	88	86	—	—	—	—	—	—	—	86
Nucleus vestibularis	—	—	—	—	—	—	—	—	70	14	46	—	—	—	—	43
Pyramids	13·3	12	11·6	—	—	—	—	—	—	—	—	—	—	—	—	12·3
Pes pedunculi	—	—	—	—	—	—	—	—	13	40	12	—	—	—	—	22
Internal capsule, anterior part	—	—	—	—	—	—	—	—	35	44	34	—	—	—	—	38
Internal capsule, central part	—	—	—	—	—	—	—	—	35	48	36	—	—	—	—	40
Internal capsule, posterior part	—	—	—	—	—	—	—	—	26	14	41	—	—	—	—	27
Corpus callosum	28	30	19	—	—	—	—	—	—	—	—	—	—	—	—	26
Funiculi gracilis and cuneatus	10	6	13	—	—	—	—	—	—	—	—	—	—	—	—	10
Tractus olfactorius	—	—	—	—	—	35	50	57	—	—	—	—	—	—	—	47
Nervus opticus	—	—	2·2	6·8	—	11·5	—	—	—	—	—	—	—	—	2	5·6
Chiasma opticum	—	—	—	—	—	35	—	—	—	—	—	—	—	—	—	35
Tractus opticus, whole	43	21	41	41	—	52	—	—	—	—	—	—	—	10	—	35
Tractus opticus, latero-ventral part	—	—	—	—	—	—	—	—	—	—	6·4	21	—	—	8	12
Tractus opticus, medio-dorsal part	—	—	—	—	—	—	—	—	—	—	43	39	—	—	42	41
Pituitary, anterior lobe	—	—	—	—	—	—	—	—	—	0·6	18	0	3	4	—	6
Pituitary, posterior lobe	—	—	—	—	—	—	—	—	—	0	23	2·4	9	0	—	7
Nervus vestibularis	—	—	—	—	—	—	—	—	46	20	21	—	—	—	—	29
Nervus facialis	—	—	—	—	—	—	—	—	130	96	54	—	—	—	—	93
Anterior roots	—	—	—	—	184	258	215	286	—	—	—	—	—	—	—	236

It is also interesting that the enzyme concentration in the superior colliculi and lateral geniculate bodies, which are both optic centres, is similar and higher than in the corresponding acoustic centres, the inferior colliculi and medial geniculate bodies.

The results for the optic tracts and the chiasma need some comment. The activity of the chiasma was found to be higher than that of the optic nerve and lower than that of the tracts (Exp. 6). Since no other instance is known in which an uninterrupted tract changes its enzymatic activity during its course, it seemed likely that the increase in enzymatic activity on approaching the geniculate bodies was due to an admixture of cholinergic fibres not belonging to the optic tract. Dr J. D. Green and Dr G. W. Harris, from the Anatomy School, Cambridge, whose advice we sought on the subject, suggested the supraoptic commissural fibres as a possible source of contamination. They further advised us to split the tracts longitudinally, so as to separate the dorso-medial from the ventro-lateral fibres. If the commissural fibres, most of which run in the dorso-medial part of the optic tracts, were responsible for the high enzymatic activity of the tracts, such a procedure should produce two bundles of which the ventro-lateral one should have the lower activity of the two and one which approaches that of the optic nerve. The figures of Exps. 11, 12 and 15 confirm this interpretation and suggest that the commissural fibres are cholinergic.

The figures in Table 1 show further that the nuclear masses in which sensory fibres end (the posterior horns, nuclei gracilis and cuneatus and nucleus vestibularis) have a much higher enzymatic activity than the fibres themselves but that the activity is far smaller than that of the motor nuclei of either cord or medulla.

Finally, the anterior and posterior lobes of the pituitary are found to contain very little if any enzyme for the synthesis of acetylcholine.

DISCUSSION

The examination of the different parts of the central nervous system for their power to form acetylcholine was carried out in the hope of obtaining new evidence about the role of acetylcholine as transmitter at central synapses. Different authors hold different views on this subject and the question is anything but settled. The opposing views have been reviewed by Feldberg (1945*b*). In his opinion acetylcholine may well be the transmitter at some central synapses but not at others. The findings of the present experiments point in the same direction.

The interpretation of our results will be based on the following conceptions which are largely derived from our knowledge of the properties of cholinergic neurons in the peripheral nervous system:

(1) A cholinergic neuron is characterized by the fact that it is able to synthesize acetylcholine, not only at its ending but along the whole course of

its axon. Thus a tract composed of cholinergic fibres will be identified by a high concentration of the enzyme which synthesizes acetylcholine. Although there is at present no evidence that the enzyme has any function to fulfil along the course of the fibre, recent observations of Weiss (1947) offer an explanation for its uniform distribution in the neuron. Weiss showed that the axoplasm of nerve fibres migrates in a slow but constant flow from the cell body to the nerve endings, presumably carrying down to the synapse all enzymes manufactured in the cell.

(2) The concentration of acetylcholine-forming enzyme in a tract of fibres will be interpreted as a measure of the number of cholinergic fibres contained in the tract, and not as an indication that some fibres can produce more and others less acetylcholine per unit of time.

(3) A bundle of non-cholinergic fibres may be identified by the nearly complete lack of the enzyme which synthesizes acetylcholine. A small concentration of the enzyme need not necessarily be taken as proof of contamination with some cholinergic fibres, but may be due to the fact that practically every tissue has a slight ability to synthesize acetylcholine under the conditions of our experiments.

(4) The presence, in regions of grey matter, of a high concentration of the enzyme which synthesizes acetylcholine may indicate one of three possibilities. Either the neurons of which the nerve cells form a part, or the fibres terminating at these cells, or both, may be cholinergic. Therefore, a high concentration of enzyme in a nucleus does not necessarily imply that the enzyme is produced by the cell body or its axon. For the peripheral nervous system, this fact is illustrated by the sympathetic ganglia. They are rich in enzyme which synthesizes acetylcholine, but the enzyme is provided by the preganglionic endings and disappears after their degeneration.

(5) In interpreting the results we occasionally make use of our knowledge of the central actions of acetylcholine. It is necessary to emphasize that the sensitivity of a nerve cell does not depend on whether or not it gives rise to a cholinergic axon. Again the cells of the sympathetic ganglia demonstrate this fact. Though sensitive to acetylcholine, these cells are part of an adrenergic neuron. The reverse conclusion, however, that cells which respond to acetylcholine are cells upon which cholinergic neurons impinge, is more likely to be generally true.

With these facts in mind an interpretation of our results has been attempted.

In the voluntary motor pathway, the finding of a high enzyme concentration in the anterior horns and the motor nuclei of the cranial nerves is in agreement with the conception of a cholinergic nature of the lower motor neuron. On the other hand, the low values encountered in the pyramidal tracts indicate that the upper motor neuron is not cholinergic. In the motor area of the cerebral cortex, an intermediate value was found. This might suggest that cholinergic neurons converge on the pyramidal cells, a possibility which is strengthened by the fact that both eserine and acetylcholine, locally applied to the motor cortex, initiate impulses in the pyramidal tracts (Miller, Stavraky & Woonton, 1940). It is, however, possible that the acetylcholine, when applied artificially to the cortex or when released and allowed to accumulate after eserine, does not act on Betz's cells directly but on another type of cells, the axons of which end on the pyramidal cells.

We have seen that the voluntary motor pathway consists of a non-cholinergic upper and a cholinergic lower neuron. The fibres of the pyramidal tract, however, are not the only ones to end on the anterior horn cells. Various fibres from other systems, some of which may be cholinergic, terminate at these cells. The same cell may therefore be impinged upon by cholinergic and non-cholinergic fibres.

The succession of alternating non-cholinergic and cholinergic neurons is not confined to the motor pathway but may occur more generally in the central nervous system. Another example is provided by the optic pathway. The retina contains large amounts of the enzyme which synthesizes acetylcholine, whereas the optic nerve is practically free from it (Feldberg & Mann, 1945, 1946). This suggests that acetylcholine is the chemical transmitter at one or more of the synaptic junctions in the retina. Among these junctions may be that to the ganglionic layer from which the non-cholinergic optic nerve originates. The optic fibres end mainly in the lateral geniculate body, and the high values found for this region may indicate a cholinergic nature of the third neuron in this pathway. Further evidence on this point would have required the analysis of samples from the tract connecting the geniculate body with the visual area. This was not attempted on account of technical difficulties.

Some fibres of the optic tracts end in the superior colliculi. It may be that the high values found in this region again indicate a change-over from the second non-cholinergic to a third cholinergic neuron, but they may also result from the presence of fibres belonging to other systems.

Our results also give some information on the sensory pathway which starts in the posterior roots. These fibres are non-cholinergic as they contain practically no enzyme which synthesizes acetylcholine (Feldberg & Mann, 1945, 1946). This is in keeping with the low values now found in the central continuation of these fibres, the funiculi gracilis and cuneatus. However, the relatively high values obtained for the nuclear masses in which these sensory fibres as well as those from the eighth cranial nerve end (the posterior horns, the nuclei gracilis and cuneatus and the nucleus vestibularis) might be evidence for the cholinergic nature of the second neuron in the sensory pathway. The fibres originating from the nuclei gracilis and cuneatus terminate in the thalamus. The third neuron originating from here and leading to the cortex is probably non-cholinergic, as suggested by the low values found for the fibres of the internal capsule. In fact, the lowest values were found in its posterior part which, at least in man, is said to contain the sensory thalamic radiation.

From the low values found in the cerebellar cortex we may conclude that this region contains few, if any, cholinergic neurons. The values for the cerebellar peduncles are higher than those for the cortex and those for the region of the cerebellar nuclei are intermediate between those for the cortex and for the peduncles. Since the upper cerebellar peduncles, which largely consist of axons from the cerebellar nuclei, contain a certain amount of cholinergic fibre, it is probable that the latter fibres also originate in the central nuclei. Under those circumstances we must assume that certain of the neurons originating from the cerebellar nuclei are able to form acetylcholine. That the enzyme concentration in the nuclear region is none the less lower than in the upper peduncles can obviously largely be attributed to the ending, in the central nuclei, of the many non-cholinergic axons from the Purkinje cells.

The enzyme concentration in the caudate nucleus which belongs to a group of basal ganglia which inhibit involuntary impulses to the skeletal muscles is one of the highest encountered. The fact that administration of atropine in Parkinsonism can partly compensate for the loss of these centres is interesting in this respect, although it is not possible at the moment to offer any explanation, since the mechanism of the inhibitory action of these centres is anything but understood.

The low values found for the posterior pituitary show that this nervous structure contains only non-cholinergic nerve fibres. The high figures for the supraoptic nucleus therefore do not suggest that cholinergic fibres originate from the cells in this region but that such fibres terminate in synaptic connexion with these cells. Their sensitivity to acetylcholine, demonstrated by direct physiological evidence (Pickford, 1947), is an illustration, in the central nervous system, of acetylcholine-sensitive cells from which non-cholinergic fibres emerge. In contrast, the hypoglossal nuclei provide an instance of central nerve cells, which also have been shown to be sensitive to acetylcholine (Miller, 1943), but are the origin of cholinergic fibres. In the peripheral nervous system, these two cell types are illustrated by the cells of the sympathetic and parasympathetic ganglia, both kinds of cells being sensitive to acetylcholine, the former being the origin of non-cholinergic (adrenergic), the latter of cholinergic fibres.

The distribution of the enzyme which synthesizes acetylcholine corresponds, as far as data for comparison are available (see MacIntosh, 1941; Feldberg, 1945*b*), to the distribution of acetylcholine in different parts of the nervous system. The low acetylcholine content of the pyramids, the afferent fibres in the dorsal tracts, the cerebellum, the posterior roots and the optic nerve, and the high values for the retina and basal ganglia, suggest that the acetylcholine content of a tissue is related to, and dependent on, its ability to synthesize acetylcholine, a conclusion also reached by Feldberg & Mann (1945; 1946). There is no such striking correspondence between the distribution of cholinesterase and the enzyme for the formation of acetylcholine. This may be due to the fact that there are no reliable data for comparison. In those data which are available, no distinction has been made between true and pseudo-cholinesterase although the former alone appears to be concerned with the acetylcholine metabolism (Hawkins & Mendel, 1947). In addition, many of the subdivisions used in the examination of the distribution of cholinesterase are of no great value for a neurological approach of the kind attempted in this paper. Nevertheless, there are some data which are comparable and, in fact, show some parallelism. The high values obtained for the cholinesterase activity of the caudate nucleus and the retina correspond to the high values found in the same structures for the enzyme which synthesizes acetylcholine. On the other hand, the cholinesterase values for the anterior roots and the thalamus are only about twice as high as those found for the optic nerve and the posterior roots.

If our assumptions based on the distribution of the enzyme which synthesizes acetylcholine in the nervous system are correct, i.e. that cholinergic neurons form only a fraction of the central neurons, that along one pathway there is often a succession of alternating cholinergic and non-cholinergic neurons, and that, furthermore, the same cell may be impinged upon by endings from cholinergic and non-cholinergic neurons, it is not surprising that the attempts at demonstrating the role of acetylcholine for synaptic transmissions within the central nervous system by electrical and other methods have yielded contradictory results.

How far the interpretation of our findings is justified can only be found out by further experiments and particularly by an even more detailed analysis of the distribution of the enzyme which synthesizes acetylcholine. Degeneration experiments may help in such an analysis. One difficulty, however, will not easily be overcome, i.e. the interpretation of the intermediate values found in so many regions and the possibility that cells and fibres of a different nature intermingle in the same region. There are some other facts not easily explained at present, as, for example, the individual variations and the variations between different species. When comparison is made of the power of the nervous tissue to synthesize acetylcholine in different dogs, individual variations amounting to over 100% are found. They may in part be associated with the fact that dogs of any race were used for our experiments. They may also be related to the age of the animals. Feldberg (1945*a*) has found, for instance, that in guinea-pigs the concentration of acetylcholine in the brain tissue appears to increase with age. The species differences are even more difficult to explain, particularly the diminution in the ability to form acetylcholine shown by the more highly developed brains. We do not know how far this decrease can be accounted for by a greater proportion of supporting tissue in the more differentiated brains, or by a greater proportion of non-cholinergic neurons, or how far it could be explained on the assumption that the transmission by acetylcholine is a more primitive type of central transmission, the importance of which recedes in higher animals where it is retained in certain synapses only. These considerations emphasize the need of great caution in drawing far-reaching conclusions from data derived from one species only.

SUMMARY

1. The distribution of the enzyme or enzyme system which forms acetylcholine in the central nervous system of the dog has been determined by examining over forty separate regions with the use of a very sensitive quantitative method for the estimation of acetylcholine synthesis.

2. An attempt has been made to differentiate between cholinergic and non-cholinergic neurons in the central nervous system.

3. The results indicate that some at least of the motor and sensory pathways consist of chains of neurons which are alternately cholinergic and non-cholinergic in character.

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THE ASSAY OF THE NUTRITIVE VALUE OF A PROTEIN BY ITS EFFECT ON LIVER CYTOPLASM

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Addis and his collaborators were the first to examine in some detail the relationship between dietary protein intake and the protein content of the liver. Addis, Lee, Lew & Poo (1940) found that liver protein increased with rising casein intake. Yuen, Poo, Lew & Addis (1940) showed that serum albumin, serum globulin or yeast protein were less effective in raising liver protein than colostrum protein, casein and lactalbumin which, in turn, were inferior to liver and kidney proteins.

Kosterlitz (1944) showed that the quantity of cytoplasm present in the liver cell was dependent on the quality and quantity of dietary protein, and suggested that the estimation of labile liver cytoplasm may lend itself to a rapid assessment of the biological or nutritive values of proteins. Since that time, various procedures which may achieve this have been examined in detail with successful results (Kosterlitz, 1944–6; Kosterlitz & Campbell, 1945, 1946). Harrison & Long (1945) also followed up this suggestion and fully confirmed and extended the data given in our previous papers. Their method was based on the regeneration of protein of rats' livers following a 48 hr. fast.

METHODS

Analytical methods. These have already been described in a previous paper (Kosterlitz, 1947).

Experimental procedures. For each experiment 4 or 5 female rats, 3·75–4·5 months old, were selected from the colony. Their average body weight was 221 g., ranging between 181 and 260 g. The mean body weight of each group of 4 or 5 rats varied between 210 and 230 g. If two proteins were compared with each other, litter mates were equally distributed between the two test diets; if a protein was compared with casein, for which a regression line had been constructed, not more than 2 rats of any one litter were placed on the test diet, in order to prevent the results from being unduly biased by a particular litter.

Three different procedures were employed. In procedure 1 the rats were transferred from the stock diet directly to the test diet which was given for 7 days. The basal diet consisted of 2% agar, 3% salts, 25% sucrose, 60% potato starch and 10% lard (Kosterlitz, 1947). The lard was supplemented with vitamins A, D, and E, and the B vitamins were given partly in the form of the pure chemical compounds and partly as liver concentrate. In procedure 2 the rats were given the protein-free basal diet for 4 days, in order to deplete the liver of its 'labile' cytoplasm, and were then given the test diet for 4 days. Finally, procedure 3 had the same general arrangement as procedure 2, but no liver concentrate was given and 0·5 ml. containing the following water-soluble vitamins was given daily: 5 μg. biotin (Merck), 5 μg. folic acid (Folvite, Lederle), 20 μg. synkavit dicalcium phosphate (Roche), 30 μg. aneurin, 30 μg. pyridoxin, 50 μg. riboflavin, 100 μg. calcium *d*-pantothenate, 100 μg. nicotinic acid, 100 μg. *p*-aminobenzoic acid, 1 mg. inositol and 17·3 mg. choline hydrochloride.

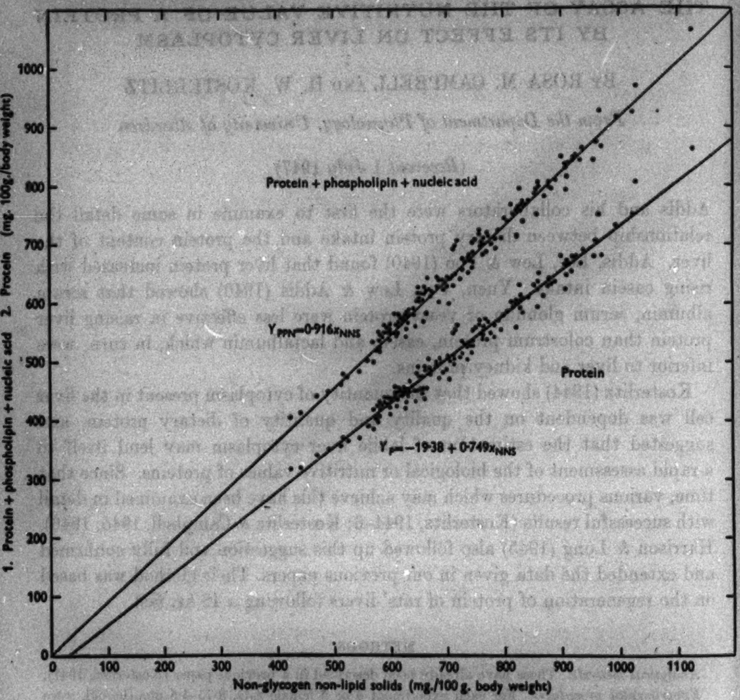

Fig. 1. Regression of protein + phospholipin + nucleic acid (y_{PPN}) and of protein (y_P) on non-glycogen non-lipid liver solids (x_{NNS}).

Estimation of 'labile liver cytoplasm'. The amount of cytoplasm present in the liver may be determined by estimating the protein, phospholipin or ribonucleic acid contents (Kosterlitz, 1944, 1947). The methods available for these estimations, although not difficult, are rather time-consuming. Harrison & Long (1945) used the total N content of the livers as an estimate of their protein contents, but this is open to certain objections. By determining the non-glycogen non-lipid solids (NNS) of the livers, values are obtained which, in female rats 4 months old, show a very high correlation with the protein + phospholipin + nucleic acid contents (PPN) and only a slightly less high correlation with the true protein values (P) (Fig. 1). The variable x_{NNS} accounts for

97·9% of the variance of y_{PPN} and for 97·2% of the variance of y_P. The standard deviation of Y_{PPN} predicted for values of $x_{NNS} = 725 \pm 100$ varies between 1·42 and 1·79 and that of Y_P between 1·35 and 1·71. Thus, in female rats of the strain used in these experiments, NNS are an excellent predictor for protein and for protein + phospholipin + nucleic acid. Labile NNS represent the difference between total and non-labile NNS; the latter are constant for rats of a given age and sex.

The estimation of NNS is technically relatively simple: the glycogen and the total fatty acids (including unsaponifiable matter) are deducted from the total solids. The total solids can be determined with a high degree of accuracy and any errors made in the estimations of glycogen and total fatty acids have little effect on the final results since, under the conditions of these experiments, total fatty acids and glycogen account for only a small part of the total solids, viz. 10–15 and 10–25% respectively. Further, with this method, information is obtained regarding the effects of various dietary proteins on the fatty acid and particularly the glycogen contents of the livers.

Calculation of results. All results are given for 100 g. initial body weight; this is calculated as the mean of the weights found on the first day of the test and the 2 days before it.

The nutritive value (NV) of a protein was computed as follows. From the regression equation for casein, the amount of casein N was calculated which would have produced the same quantity of (total) NNS as did the test protein N actually fed. The NV of the test protein was then mg. casein N/mg. test protein N. If the mean daily food intake was below 7 g./100 g. body weight, a correction of +46 mg. NNS/g. food deficit had to be applied (cf. Table 2). As will be shown later in this paper, a reduction in calorie intake causes, by itself, a decrease in NNS. It should be noted that the term 'nutritive value' (NV), as used in this paper, corresponds to the 'net utilization value' and not to the 'biological value' of the balance-sheet method (Block & Mitchell, 1946–7). NV and 'net utilization value' are calculated in terms of ingested protein N, and 'biological value' in terms of protein N actually absorbed.

Statistical treatment of the results. The variances of NNS within groups receiving the same type of diet did not differ significantly among themselves, with the exception of the groups receiving the stock and flour diets. The variances within the groups other than cereal and stock diets were pooled to give a combined estimate of 1348·2, obtained with 173 degrees of freedom. The variance within the groups receiving flour diets by procedure 1 and 2 was 2359 (36 degrees of freedom) and within the groups receiving flour diets by procedure 3, 504 (16 degrees of freedom). So far, no explanation can be offered for these differences in variability. The variance within the group receiving the stock diet was 4329 (19 degrees of freedom), very significantly larger than the variance of the non-flour diets. This was possibly due to the fact that the food intake of the rats on the stock diet was not controlled.

If not expressly stated otherwise, the symbols used in the regression equations of this paper are as follows: Y stands for predicted (total) NNS (mg./100 g. body weight), x for the logarithms of the daily protein N intakes (mg./100 g. body weight), and t for days on the protein-free diet.

RESULTS

Changes in the composition of the liver caused by varying the intake of protein

In the experiments in which varying quantities of casein were given by procedure 1, the mean daily food intake varied between 6·65 and 7·3 g./100 g. body weight with an overall mean of 7·0 g. In order to save space, the results are not given in detail; they may be summarized as follows. The weight and the water content of the livers did not show any significant changes when the casein N intake varied between 50 and 280 mg./100 g. body weight, while both values increased when the casein N intake was between 380 and 760 mg./100 g. body weight. The protein and phospholipin contents, and to a less extent the nucleic acid content, increased with rising casein N intake. There were no significant changes in the glycogen and neutral lipid contents of the livers. The phospholipin indices (phospholipin × 100/protein + phospholipin + nucleic acid) fell slightly, and the protein indices rose slightly, when the protein intake was above 200 mg. N/100 g. body weight, but otherwise showed no significant changes. The nucleic acid indices decreased with increasing protein intake; this is in agreement with the view that only ribonucleic acid takes part in these changes (Kosterlitz, 1944, 1947; Campbell & Kosterlitz, 1947). Substitution of part of the carbohydrate of the diet by fat did not lead to any important changes in the composition of liver tissue. Apart from a slight increase in the neutral lipid content, the results obtained by feeding 5·75 g./100 g. body weight of a 30 % casein diet containing 40 % lard were not significantly different from those obtained by feeding the diet containing only 10 % lard.

Diets deficient in tryptophan led to considerable losses in protein, phospholipin and nucleic acid (labile cytoplasm) from the liver. If appetite was markedly depressed, the liver weight fell simultaneously as in the case of acid-hydrolysed casein. If, on the other hand, the rats ate comparatively well, as, for example, in the case of zein, increased amounts of liver glycogen were deposited and the liver weight was only slightly diminished. Deficiency of lysine was very much less effective in decreasing labile liver cytoplasm than was tryptophan deficiency.

It has been shown previously (Kosterlitz, 1944, 1947) that rats fed on a protein-free diet for 4 days lost practically all labile liver cytoplasm. Addition of 0·6 % *dl*-methionine or of 0·38 % *l*-tryptophan did not prevent this. There was, however, an unexpected increase in the glycogen and neutral lipid contents of the livers when methionine was added to the basal protein-free diet. When tryptophan was added, the neutral lipid content increased but the glycogen content was smaller than in the rats fed on the basal protein-free diet only. The liver weights showed corresponding changes.

Disappearance of labile liver cytoplasm in protein deficiency

Further observations on the relation of NNS to time in female rats transferred from the stock diet to a protein-free diet indicate that the loss of NNS during the first few days is better described by a simple exponential function of time than by the combined exponential and linear function of Campbell & Kosterlitz (1946). The recalculated equation is

$$Y = 620 + 254\,(0{\cdot}33)^t,$$

the first term on the right representing non-labile NNS (mg./100 g. body weight) and the second the amount of labile NNS after *t* days on the protein-free diet (Fig. 2). It follows that the labile NNS falls to 9·1 mg./100 g. body weight after 3 days and to 3 mg./100 g. body weight after 4 days on the protein-free diet.

Diets deficient in single essential amino-acids, or in calories, were less efficient in removing labile NNS than was a protein-free diet (Fig. 2). If rats had lost their labile NNS by being fed on a protein-free diet, 2 days of refeeding with a 20% casein diet almost completely restored their labile NNS (Fig. 3). For this reason, in procedure 2, rats were first fed on a protein-free diet for 4 days in order to free their livers of labile NNS, and then given the test diet for 4 days. No experiments were specially designed to ascertain whether, in procedure 1, the duration of 7 days on the test diet was sufficient to ensure equilibrium of labile NNS. However, as will be shown later, the regression coefficients for casein obtained by procedures 1 and 2 did not differ significantly, and in the tests with flour proteins no difference was found between the two procedures.

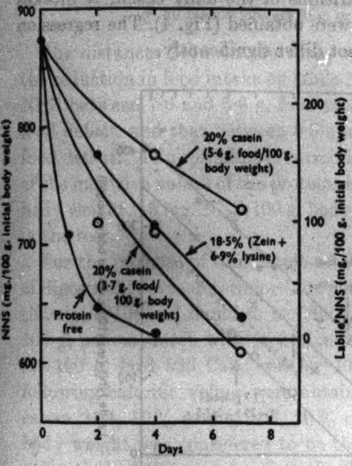

Fig. 2.

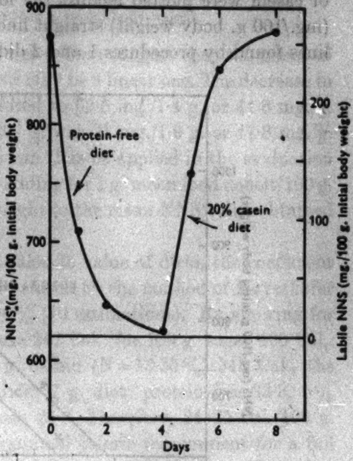

Fig. 3.

Fig. 2. Rate of disappearance of labile liver cytoplasm. The curves were drawn by eye with the exception of that for the protein-free diet, which fits $Y = 620 + 254\,(0{\cdot}33)^t$. Each point represents the mean of at least 4 rats.

Fig. 3. Rate of breakdown and formation of labile liver cytoplasm. Each point represents the mean of at least 4 rats.

Although, on theoretical grounds, procedure 2 may have been expected to be more sensitive than procedure 1, no such difference was found. From a practical point of view, procedure 2 was superior to procedure 1 in that less protein was required for the test and, in the case of deficient and unpalatable diets, the rats were more likely to eat their daily ration.

Quantitative relationships between casein intake (at different levels of food intake) and NNS

Casein was chosen as a standard for the evaluation of the nutritive values of proteins. Although it is not a complete protein, it is readily available in sufficiently pure grade.

When the NNS (mg./100 g. body weight) of rats fed on different quantities of casein were plotted against the logarithms of the daily casein N intakes (mg./100 g. body weight) straight lines were obtained (Fig. 4). The regression lines found by procedures 1 and 2 did not differ significantly.

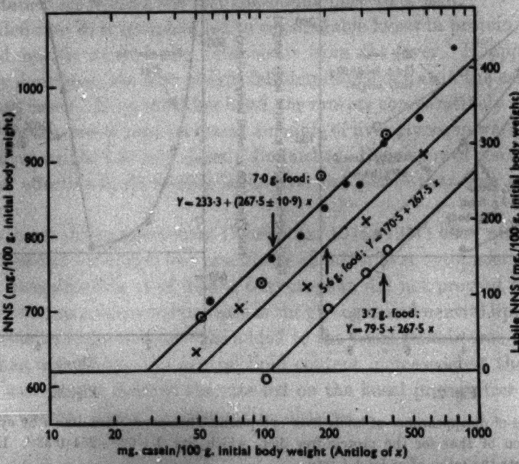

Fig. 4. Regression lines for casein for different calorie intakes. Each point represents the mean of at least 4 rats. ●, 7 g. food/100 g. body weight (procedure 1); ⊙, 7 g. food/100 g. body weight (procedure 2); ×, 5·6 g. food/100 g. body weight (procedure 1); ○, 3·7 g. food/100 g. body weight (procedure 1).

When the daily food intake was lowered from 7·0 to 5·6 or 3·7 g./100 g. body weight, the regression coefficient did not alter, but the NNS values obtained for a given casein intake fell with decreasing food intakes. The three lines thus obtained differed very significantly as regards vertical distance. If the NV of casein at 7·0 g. food intake is taken as 1·00, the apparent NV's at 5·6 and 3·7 g. food intake are 0·58 and 0·26 respectively. On the other hand, at 8·5 g. food intake the apparent NV is the same as at 7·0 g. so that 7·0 g. may be taken as the minimal food intake which will assure full utilization of the proteins (Table 1).

TABLE 1. Effect of feeding 5 and 10% casein diets at the rate of 26 or 30 Cal./100 g. body weight on the non-glycogen non-lipid liver solids (procedure 1)

		Mean daily intake of			
Diet	No. of rats	Food (g.)*	Casein N (mg.)	Non-glycogen non-lipid liver solids (NNS) (mg.)	Values calculated from regression line (mg.)
5% casein	4	7·3	55	731 ± 19·8	699 ± 7·4
5% casein	4	8·45	61	691 ± 19·8	710 ± 7·0
10% casein	3	7·45	101	755 ± 22·9	769 ± 5·5
10% casein	4	8·5	118	781 ± 19·8	788 ± 5·1

* All estimations are in g. or mg./100 g. initial body weight.

The distances between the three regression lines suggest that the effect of the reduction in food intake on labile NNS may be a linear one. The decrease in NNS between 7·0 and 5·6 g. food amounted to 62·8 mg./1·4 g. or 44·8 mg./g. food deficit, and that between 5·6 and 3·7 g. was 91 mg./1·9 g. or 47·8 mg./g. food deficit. An approximate correction can thus be applied in the evaluation of the nutritive values of the proteins by adding for 1 g. mean food deficit/100 g. body weight, 46 mg. NNS/100 g. body weight to the mean NNS value obtained in the test.

For the purpose of calculation of the calorific value of diets, the coefficient of digestibility was determined for potato starch by the method of Mayerhofer (König, 1910) and found to be 68·9 ± 2·6% (10 estimations). By allowing for 100 g. potato starch with 18·2% moisture 242 Cal., for 100 g. sugar 400 Cal., for 100 g. lard 925 Cal., and for 100 g. casein (N = 13·35%) 348 Cal., the following calorific values were obtained for 7 g. diet: protein-free 23·8, 5% casein 24·4, 10% casein 24·6, 20% casein 25·3. Therefore, 24–25 Cal./100 g. body weight was considered to be the minimal calorie requirement for a full utilization of the proteins fed. (In a preliminary communication (Kosterlitz & Campbell, 1946) the minimal calorie requirement was erroneously given as 27–30 Cal./100 g. body weight.) It is not possible to give an estimate of the calorific value of the high-casein diets as the digestibility of the casein has not been determined. In any case, any decrease in the digestibility of casein would probably be offset by the smaller amount of poorly digestible potato starch in the high-casein diets.

Effects of nitrogenous constituents of liver extract and potato starch

In procedures 1 and 2, small quantities of amino-acids may be introduced by the liver concentrate. Therefore, 12 animals were fed by procedure 3 in which the water-soluble vitamins were given as pure chemical compounds. At the same time, the potato starch was replaced by sucrose. The concentrations of casein in the diet were 10, 23 and 54% (Fig. 5). The regression coefficient of 327·7 ± 41·8 was not significantly different from that of the casein lines obtained previously. The difference in vertical distance between this line and that for casein with liver extract and potato starch is 31·2 ± 11·6, which is significant at the 1% level. These results suggest that amino-acids in the liver concentrate and possibly also the potato starch raise the nutritive value of casein above its true figure.

The nutritive values (NV) of various proteins

Egg albumin (procedure 1). In the case of egg albumin, sufficient values (16) were available to construct a regression line and compare it with that obtained for casein (Fig. 5). The regression coefficient was found to be 253·0 ± 29·4, which did not differ significantly from that of casein, 267·5. The difference in vertical distance was 28·5 ± 10·24, which was significant at the 1% level. The calculation of the nutritive value gives NV = 1·28, indicating that egg albumin is significantly superior to casein.

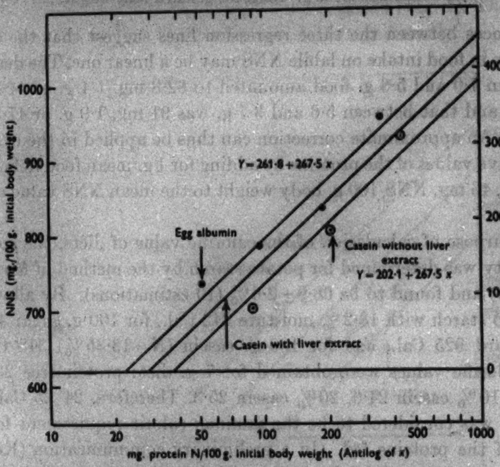

Fig. 5. Regression lines for egg albumin, casein with and without liver extract. Each point represents the mean of at least 4 rats.

Zein (procedures 1 and 2). The results with zein, zein supplemented with either tryptophan or lysine or with both, are summarized in Tables 2 and 3. Insufficient data are available to construct regression lines in these cases. Zein was tested at the 18 and 55% levels of intake, and NV's of 0·29 and 0·22 respectively were obtained. Since the difference between the two NV's was not significant, a pooled NV of 0·25 was obtained. Supplementation with lysine alone did not affect the NV, yielding a value of 0·23. Supplementation with both tryptophan and lysine raised the NV to 0·89, which is not significantly different from that of casein. Supplementation with 1·82% *l*- or *dl*-tryptophan

TABLE 2. Estimation of non-glycogen non-lipid liver solids and assessment of the nutritive value of zein with and without the addition of tryptophan and lysine (procedure 1)

Diet	No. of rats	Change in weight during exp. (g.)	Mean daily intake of		Total solids (mg.)	Glycogen (mg.)	Total fatty acids (mg.)	Non-glycogen non-lipid liver solids (NNS)		Daily casein N required to produce effect of test protein (mg.)	Nutritive value of test protein (significance of difference from casein = 1)
			Food (g.)*	N (mg.)				Found (mg.)	Corrected for inadequate food intake (mg.)		
18·5% zein	4	−10	6·65	183	978	152	146	680 ± 18·1	696	53·7	0·29 (SS)†
55·5% zein	4	−14	6·6	533	999	116	116	767 ± 18·1	785	115·7	0·22 (SS)
18·5% zein supplemented with 6·9% *l*-(+)-lysine hydrochloride	4	−17	6·45	178	871	118	115	638 ± 18·1	663	40·4	0·23 (SS)
18·5% zein supplemented with 1·82% *l*-tryptophan	4	− 7	7·0	192	1065	166	139	760 ± 18·1	—	93·1	0·49 (SS)
18·5% zein supplemented with 1·82% *l*-tryptophan and 6·9% *l*-(+)-lysine hydrochloride	4	− 3	7·0	191	1028	65	133	830 ± 18·1	—	169·8	0·89 (NS)

* All estimations are in g. or mg./100 g. initial body weight. † (NS), not significant; (SS), significant at the 1 % level.

TABLE 3. Assessment of the nutritive value of zein supplemented with 1·82% *dl*-tryptophan (procedure 2)

Concentration of zein supplemented with 1·82% *dl*-tryptophan in diet	No. of rats	Changes in weight during experiment (g.)	Mean daily intake of		NNS (mg.)	Nutritive value of test protein (significance of difference from casein = 1)
			Food (g.)*	N (mg.)		
4·6	4	−7† +2	7·5 7·45	3·3 56	676 ± 18·1	0·82 (NS)‡
9·3	5	−9 +1	7·35 7·35	3·5 104	748 ± 16·2	0·81 (NS)
18·5	5	−13 +7	7·4 6·9	3·3 188	758 ± 16·2	0·51 (SS)
30·0	4	−4 −3	7·35 5·9	3·4 266	812 ± 18·1	0·84 (NS)

* All estimations are in g. or mg./100 g. initial body weight.

† The values above the horizontal line were obtained during the protein-free period and those below the line during the test period.

‡ (NS), not significant; (SS), significant at the 1 % level.

alone raised the NV to 0·5 when the diet contained 18·5% zein. On the other hand, at zein levels of 4·6, 9·3 and 30%, supplementation with 1·82% *dl*-tryptophan raised the NV to approximately 0·8. Because of the small number of animals used in the tests at the different levels of supplemented zein intake, the difference between 0·5 and 0·8 is just significant at the 5% but not at the 1% level. If, in Table 3, all NV values are pooled, a combined value of 0·736 is obtained which is very significantly lower than the NV of casein. If only the values for 4·6, 9·3 and 30% zein, supplemented with tryptophan, are pooled for the estimation of NV, a value of 0·822 is found which is just significantly lower (at the 5% level) than that of casein. Further, the values given in Table 2 for zein supplemented by tryptophan alone and for zein supplemented by tryptophan and lysine differ very significantly in favour of the latter. From these results it may be concluded that tryptophan deficiency causes a very marked lowering of NV, while lysine deficiency has a very much smaller but probably still significant effect.

Soya bean proteins (procedure 1). The isolated soya bean proteins had a surprisingly low NV of 0·38, which was only slightly raised to 0·48 by supplementation with 1·2% *dl*-methionine (Table 4). Because of the small number of animals used, this difference is not statistically significant. The smallest difference in NV which would have been significant is 0·14.

Protein hydrolysates (procedure 1). An acid hydrolysate of casein which had a tryptophan content of only 0·3% had a NV of 0·42 (Table 4). This could be raised by supplementation with *dl*-tryptophan. Three different levels were used: 0·38 and 0·77% *dl*-tryptophan were definitely insufficient, while the hydrolysate supplemented with 1·54% tryptophan had a NV of 0·79 which did not differ significantly from the NV of casein. Another acid hydrolysate of casein supplemented with 1% *dl*-tryptophan had a NV of 1·09. It would appear that a tryptophan content of 1·2–1·5% is required in the hydrolysate, a figure which agrees well with the values obtained by the analysis of intact casein. In enzymatic hydrolysis of casein tryptophan is not destroyed; the only enzymatic hydrolysate which was tested had, however, a slightly lowered NV (0·72). A commercial enzymatic digest of meat had the same NV as casein.

Proteins of flours of different extractions (procedures 1, 2 and 3). The flours tested were samples of the 'K' series of flours used by Chick, Copping & Slack (1946). They were milled from a grist of mixed wheat. Their nitrogen contents were equalized by adding potato starch in the first and second series of experiments and by adding sucrose in the third series. Further, in the experiments with whole wheat meal, the daily food intake was raised from 7·5 to 7·85 g./100 g. body weight in order to allow for the low content of utilizable calories in whole wheat (McCance, Widdowson, Moran, Pringle & Macrae, 1945). The content of utilizable calories was well above the minimum requirements, viz. 28·5 Cal./7·5 g. diet in the case of the 70, 80 and 85% extraction flours and

28·5 Cal./7·85 g. diet in the case of the whole wheat flour. In procedure 1, the weights of the rats remained constant; in procedures 2 and 3 the recovery in body weight during the test period was not significantly affected by the different extraction rates of the flours.

TABLE 4. Assessment of the nutritive values of various proteins and protein hydrolysates (procedure 1)

Protein tested	No. of rats	Change in weight during experiment (g.)	Mean daily intake of		NNS (mg.)	Nutritive value of test protein (significance of difference from casein = 1)
			Food (g.)*	N (mg.)		
Soya bean proteins	4	− 6	7·1	196	734	0·38 (SS)†
Soya bean proteins supplemented with 1·2% *dl*-methionine	4	0	6·7	184	741	0·48 (SS)
Acid hydrolysate of casein	4	−23	5·2	144	624	0·42 (SS)
Acid hydrolysate of casein supplemented with 0·38% *dl*-tryptophan	4	−10	6·6	180	736	0·48 (SS)
Acid hydrolysate of casein supplemented with 0·77% *dl*-tryptophan	4	− 3	7·05	193	776	0·55 (SS)
Acid hydrolysate of casein supplemented with 1·54% *dl*-tryptophan	4	− 2	6·2	171	766	0·79 (NS)
Commercial acid hydrolysate of casein supplemented with 1% *dl*-tryptophan	4	− 1	7·5	204	861	1·09 (NS)
Commercial enzymatic hydrolysate of casein	5	− 7	7·3	102	733	0·72 (S)
Commercial enzymatic hydrolysate of meat	5	+ 3	7·1	195	840	0·95 (NS)

* All estimations are in g. or mg./100 g. initial body weight.

† (NS), not significant; (S), significant at the 5% level; (SS) significant at the 1% level.

No significant differences were found between the NNS values obtained by procedures 1 and 2; therefore the results of the first and second series of experiments were pooled (Table 5). None of the results differed significantly from any other, although the differences between the 70 and 85% extraction flours (33 ± 17·2 mg. NNS/100 g. body weight) almost reached significance at the 5% level. The minimum difference in NV which could have been considered significant was 25·9%. Thus the difference in NV between the 70 and 85% extraction flours can be taken to be less than 25·9%. The relatively low sensitivity of the flour tests was due to a high variance of error ($S^2 = 2359$) which was significantly greater than that for the non-flour experiments ($S^2 = 1348$).

In the third series of experiments in which 70 and 100% extraction flours were compared, procedure 3 was employed, in which the whole liver concentrate was replaced by biotin, folic acid, *p*-aminobenzoic acid and vitamin K. The difference of 5·0 ± 10·6 between the two flours was not significant; because of the low variance of error ($S^2 = 504$) in this series, a difference of 22·5 mg. NNS/100 g. body weight would have been significant. Thus, a difference in NV of 17·6% would have been detectable.

TABLE 5. Assessment of the nutritive values of the proteins of flours of different extraction rates

Extraction rate of flour (%)	No. of rats	Mean daily intake of		NNS (mg.)	Nutritive value of flour proteins (significance of difference from casein = 1)
		Food (g.)*	N (mg.)		
		First and second series combined			
70	15	$\frac{7\cdot4^{\dagger}}{7\cdot45}$	$\frac{3\cdot4}{130}$	755 ± 12·5	0·70 (SS)‡
80	10	$\frac{7\cdot25}{7\cdot35}$	$\frac{3\cdot3}{129}$	770 ± 15·4	0·79 (NS)
85	17	$\frac{7\cdot45}{7\cdot4}$	$\frac{3\cdot4}{130}$	788 ± 11·8	0·92 (NS)
		Third series			
70	9	$\frac{7\cdot3}{7\cdot4}$	$\frac{0\cdot4}{124}$	762 ± 7·5	0·77(SS)§
100	9	$\frac{7\cdot4}{7\cdot85}$	$\frac{0\cdot4}{126}$	767 ± 7·5	0·79(SS)§

* All estimations are in g. or mg./100 g. initial body weight.

† The values above the horizontal line were obtained during the protein-free period and those below the line during the test period.

‡ (NS), not significant; (SS), significant at the 1% level.

§ The nutritive values of 70 and 100% extraction flours, third series, would be raised to 1·00 and 1·02 respectively by using for calculation the regression line for casein obtained by procedure 3.

DISCUSSION

Two methods have been widely used for the evaluation of the nutritive values of proteins. The first determines, in growing rats, the maximum ratio of increase of body weight to protein consumed, known as 'protein efficiency ratio' (Osborne, Mendel & Ferry, 1919). The second method (balance-sheet method) measures the percentage of absorbed nitrogen retained for maintenance and, in young rats, for growth, known as the 'biological value' (Mitchell, 1924).

The method described in this paper has, in the first instance, been designed for the evaluation of the nutritive value of proteins in adult rats. A comparison may therefore be made with the balance-sheet method. In Mitchell's method the determination of the endogenous urinary nitrogen and of the faecal metabolic nitrogen may cause considerable difficulties and, if not done with great caution, vitiate the results. On the other hand, the standard deviation of the results is small in experienced hands; Mitchell, Burroughs & Beadles (1936) state that they can determine the biological values with a standard error of only 1·2. Statistical evaluation of results obtained from other laboratories shows that this low coefficient of variation is not always found.

In the method described in this paper, the standard deviation of the actual estimations of the NNS is reasonably small, and corresponds to a value of

±9·2 for the standard error of a mean estimated from 16 animals. This is only 1–1·5% of mean NNS values in the usual range of 700–900 mg./100 g. body weight. On the other hand, because of the logarithmic relationship between protein intake and NNS, the coefficient of variation of the NV values is considerably greater, viz. 7·4% for a mean obtained from 16 estimations.

A reduction of this relatively high variability may possibly be obtained by replacement of the liver concentrate with synthetic biotin and folic acid. This was adopted in procedure 3 and seemed to have the desired effects in the flour experiments. Further, it may be possible that the ratios of protein/deoxyribonucleic acid may have a smaller variance of error than NNS; the estimation of these ratios would, however, require rather complicated chemical techniques. Lastly, in the male rat the regression coefficient for NNS on log casein intake is about 75% greater than in the female (Campbell & Kosterlitz, 1946), so that it may be preferable to use male rats.

The relatively low sensitivity of the new method is its only serious drawback. Its advantages are the following: The technique is simple and rapid; this has also been stressed by Harrison & Long (1945). No assumptions have to be made, as, for example, in the 'balance-sheet' method, with regard to the constancy of the excretions of endogenous nitrogen; the statistical treatment of the results is therefore uncomplicated. The effect of an insufficient calorie intake on the nutritive value of a protein can be studied quantitatively. The test can be made at any level of protein intake above minimum requirement while the biological value determined by the balance-sheet method decreases with rising protein intake. Thus, it has been found to be advantageous to use protein concentrations between 10 and 20%. From a practical point of view, the new method is particularly suitable for the 'screening' of proteins or protein preparations when a rapid assay is required without need for great accuracy.

Harrison & Long (1945), following up the suggestion made by one of us with regard to the assay of the nutritive values of proteins (Kosterlitz, 1944), fasted rats for 48 hr., and then fed them for 4 days on a diet containing the protein to be tested. During this period, male rats weighing 250–280 g. were given 12 g. of a diet containing 26% fat on the first day and 10 g. on each of the subsequent 3 days. Thus, the calorie intake was considerably lower than the 24–25 Cal./100 g. body weight which may be considered as the minimum for the full utilization of proteins. For this reason, Harrison and Long's method is not quite as sensitive as our method, and diets containing 10% casein or 10% lactalbumin did not cause any 'regeneration' of liver protein. In principle, however, the results obtained by Harrison & Long and those by ourselves are in good agreement.

The nutritive values obtained with the new method agree fairly well with those obtained with the older methods. Gelatin has a very low NV (Kosterlitz, 1947). Tryptophan deficiency causes a serious fall in the NV. Acid-hydrolysed casein, which is deficient in tryptophan, has a low NV which can be raised to normal by the addition of about 1% *dl*-tryptophan.

Absence of lysine from a protein causes a decrease of the NV which appears to be just significant. According to Neuberger & Webster (1945) lysine is required for the maintenance metabolism of the adult rat, a view which is not shared by Mitchell (1947). Frazier, Wissler, Steffee, Woolridge & Cannon (1947) found that weight recovery of protein-depleted adult rats is impossible in the absence of lysine. The reduction of labile liver cytoplasm in lysine deficiency suggests that absence of this amino-acid from the diet of the adult rat interferes with its protein metabolism, although a considerable amount of labile liver cytoplasm is present even in the complete absence of lysine. It is of interest that the same amount of NNS is found in lysine deficiency whether the rats are transferred directly from the stock diet to the test diet or are first given a protein-free diet and then the test diet. In the latter case labile liver cytoplasm is built up in the absence of dietary lysine which therefore has been obtained from other tissues of the body.

No significant difference could be detected between flours of 70, 80, 85 and 100% extraction. This finding is in good agreement with those of Henry & Kon (1945); although these authors found a decrease in the biological values of the proteins of bread with decreasing extraction rates, the 'net utilization' values (= biological value × true digestibility $\times 10^{-2}$) indicate no difference between the various extraction rates. Our results on the adult rat are not necessarily in disagreement with the results obtained by Chick, Copping & Slack (1946) who, in growth experiments, found a superiority of the high extraction flours.

A comparison of the NV's of some of the proteins tested in this paper shows a fairly good agreement with the 'net utilization' values obtained by Mitchell's method (Table 6). For this purpose, the NV's have been multiplied by 68, the 'net utilization' value of casein.

TABLE 6. Comparison of net utilization values obtained by the balance-sheet method with nutritive values obtained by the liver cytoplasm method

	NV	NV × 68	Net utilization*
Egg albumin	1·28	87	83 (growing rat)
Casein	1·0	68	68 (growing rat)
Whole wheat	0·79	54	61 (growing rat) 69 (adult rat)
White flour	0·73	50	52 (growing rat)
Soya bean flour	0·38	26	72 (growing rat) 49 (adult rat)

* Block & Mitchell, 1946–7; Mitchell, 1947.

The effect of reducing calorie intake on the nutritive value of a protein is of considerable interest. It has, of course, been known for a long time that, unless sufficient calories are supplied in the diet, part of the protein will be utilized for energy requirements. Nevertheless, 'labile' NNS are still present in calorie deficiency, although with decreasing food intake more and more protein N is required to obtain the same effect. That the regression coefficient is not significantly affected by the reduction in calories means that the response of the liver to increasing protein intake is only quantitatively but not qualitatively altered. It was found that about 24 Cal./100 g. are required for a 4-month old female rat to utilize fully the protein of the diet. Reductions of the calorie intake by 20 and 47% caused a fall of the NV from 1·0 to 0·58 and 0·26, respectively; this indicates that a relatively small deficiency in calories will lead to serious protein deficiency. From the practical point of view, these findings suggest that in conditions of shortage of both protein and calories, attention should first be paid to a sufficient supply of calories. On the other hand, if, for pathological reasons, the ingestion of the normal quantities of food should become impossible, large quantities of protein will have to be administered; thus, if in the rat the food intake is reduced by half, the protein concentration will have to be raised eight times in order to obtain the quantity of NNS found when the food intake is normal.

Under the conditions of the experiments described in this paper, in which adequate quantities of choline were supplied, only minor changes were found in the neutral lipid contents of the livers, while there were great variations in the glycogen contents. The glycogen contents were often but not always raised when the NV of the test protein was low, for example in the case of zein or zein supplemented with tryptophan only. If lysine was also added, the glycogen content was lowered to values usually found in casein or stock diets. No such excessive deposition of glycogen was found when soya bean proteins of a low NV were fed. It is possible that these differences between the proteins may be due to variations in the glycogenic properties of the constituent amino-acids.

SUMMARY

1. A new method is described for the assay of the nutritive value of proteins in adult rats. It is based on the fact that the amount of labile cytoplasm present in the liver depends on the quantity and quality of dietary protein. Labile liver cytoplasm can be estimated by a determination of protein, phospholipin and nucleic acid or of the non-glycogen non-lipid liver solids (NNS).

2. The quantity of NNS varies directly with the logarithm of the protein intake. The method can therefore be used at all levels of protein intake above minimum requirement. Regression equations are given for casein with normal and reduced calorie intake, and for egg albumin.

3. A reduction in calorie intake lowered the amount of NNS without altering the regression coefficient. Decreases of 20 and 47% in calorie intake reduced the nutritive value of casein from 1 to 0·58 and 0·26 respectively. The implications of these findings are discussed.

4. Nutritive values (NV) of various proteins have been determined and found to be in fairly good agreement with those obtained by the older methods. If the NV of casein was taken to be 1·0, zein had a NV of 0·25 which was not altered by the addition of lysine. Addition of tryptophan to zein caused a marked improvement, and the NV of zein supplemented by both tryptophan and lysine was not significantly different from that of casein. Acid hydrolysates of casein had a low NV due to tryptophan deficiency; addition of 1% *dl*-tryptophan restored the NV to its original level. The NV of egg albumin of 1·28 was significantly higher than that of casein. No significant differences were found between the proteins of flours of 70, 80, 85 and 100% extraction rate.

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THE RELATIVE HUMIDITY OF THE SKIN

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The only extensive experimental data on the effects on evaporation from the skin of varying the humidity of the air are those of Winslow, Herrington & Gagge (1937). They were obtained by means of a calorimeter which allowed separate determinations of the heat exchanges by radiation, convection and evaporation, and a full account of the experimental methods and results is given in their paper and its references. In experiments on resting subjects where air movement was constant, and radiation and air temperatures were equal, it was possible to show that altering air humidity from 17 to 80% made no difference to evaporative heat loss. Also it was concluded that variations in the humidity of the air did not affect skin temperature. Nevertheless, if evaporative loss and skin temperature are to remain constant with altering air humidity, there must be some compensatory change in the physical state of the skin surface from which evaporation takes place.

Evaporative loss of water from the skin surface depends on the difference between the vapour pressure of water vapour at the skin surface and in the air:

$$E/A = k\,(v.p._s - v.p._a), \qquad (1)^*$$

where E/A is the evaporative loss per unit area of skin surface, k is a constant depending on air movement, $v.p._s$ is the aqueous vapour pressure at the skin surface, and $v.p._a$ is the aqueous vapour pressure of the air. Further,

$$v.p._a = rh_a\,\epsilon\,(T_A),$$

where rh_a = the relative humidity of the air, and $\epsilon\,(T_A)$ = the aqueous vapour pressure in air saturated with water at the temperature of the air T_A.

Gagge (1937) compared the surface of the skin to the surface of a pool of water of varying area under the same physical conditions of temperature and

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[*] The symbols used in this paper are, wherever possible, the same as those used by Winslow *et al.* (1937).

air movement as the skin. The term $v.p._s$ was taken as equal to $\epsilon(T_S)$, where $\epsilon(T_S)$ was the aqueous vapour pressure in air saturated with water at the skin temperature T_S. Therefore

$$\xi=\mu\,[\epsilon\,(T_S)-rh_a\,\epsilon\,(T_A)], \tag{2}$$

where ξ is the evaporative loss per unit area of the pool of water, and μ is the constant depending on air movement.

The evaporative losses from the body and from such a pool of water of area equal to that of the skin (A) will be the same when the whole body surface is covered by liquid perspiration, i.e. completely wet. When only a fraction of the body surface is wetted, the evaporative loss per unit area of skin will be less than ξ, and the ratio of the measured loss E/A under any set of experimental conditions to ξ gives a measure of the fraction w of the body surface which is completely wet in those conditions:

$$\frac{E/A}{\xi}=w. \tag{3}$$

Combining (3) with (2),

$$w=\frac{E/A}{\mu\,[\epsilon\,(T_S)-rh_a\,\epsilon\,(T_A)]}. \tag{4}$$

This derivation, depending on a comparison of the surface or the body with that of a pool of water of smaller area, assumes that there is no exchange of water between the atmosphere and that fraction of the body's surface which is not completely wet. This, in turn, implies that the aqueous vapour pressure at the surface of this fraction of the skin varies automatically so as to equal the aqueous vapour pressure of the air whatever the atmospheric humidity. This consequence of the method by which w is calculated suggests that it is not likely to be a useful concept, and this is confirmed by the experimental data.

In Fig. 1 are plotted the values obtained by Winslow *et al.* (1937) for the fraction w at varying air temperatures. The values obtained at different air humidities are distinguished, and it can be seen that there is little consistent variation of w with humidity. This is also shown in Fig. 1 of the paper by Gagge (1937) where air humidities varied from 34 to 67 % without altering w. It does not seem, therefore, as if w describes the physical change in the skin which is a necessary consequence of the constancy of evaporative loss with varying air humidity.

From the same experimental material of Winslow *et al.* (1937) it is possible to derive another variable, the relative humidity of the skin, which does vary in a regular manner with varying relative humidity of the air, and can be plausibly interpreted as a real property of the skin (Fig. 2).

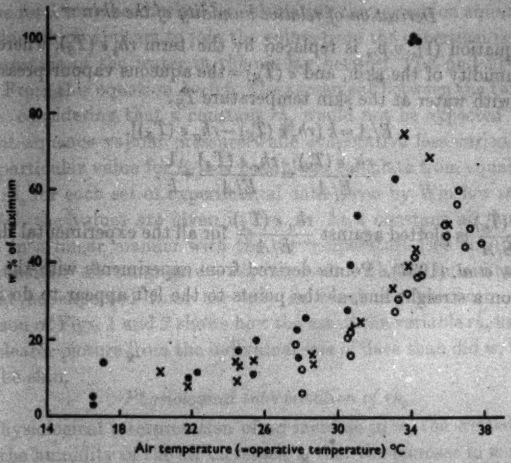

Fig. 1. The variation of *w*, the wettedness of the skin (Gagge), with air temperature as air humidity varies. Air humidity: ● 60–80%; × 30–59%; ○ 14–29%.

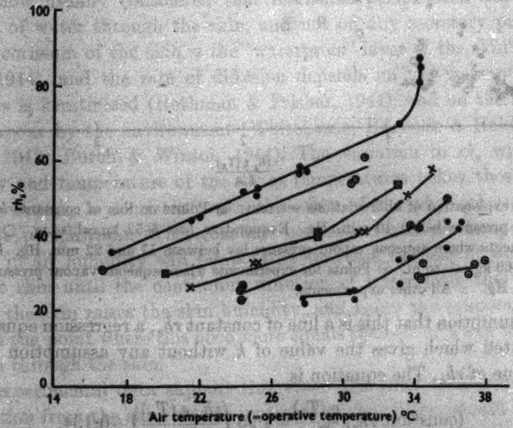

Fig. 2. The variation of rh_s, the relative humidity of the skin, with air temperature as air humidity varies. Air humidity: ● 70–80%; ◎ 60–69%; ⊠ 50–59%; × 40–49%; ⊗ 30–39%; ● 20–29%; ◎ 14–19%.

Derivation of relative humidity of the skin

If, in equation (1), *v.p.*$_s$ is replaced by the term $rh_s\,\epsilon\,(T_S)$, where rh_s = the relative humidity of the skin, and $\epsilon\,(T_S)$ = the aqueous vapour pressure in air saturated with water at the skin temperature T_S,

$$E/A = k\,[rh_s\,\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)]. \tag{5}$$

Therefore
$$\frac{rh_s\,\epsilon\,(T_S)}{E/A} = \frac{rh_a\,\epsilon\,(T_A)}{E/A} + \frac{1}{k}. \tag{6}$$

In Fig. 3 $\dfrac{\epsilon\,(T_S)}{E/A}$ is plotted against $\dfrac{rh_a\,\epsilon\,(T_A)}{E/A}$ for all the experimental data given by Winslow *et al.* (1937). Points derived from experiments with the same rh_s should lie on a straight line, as the points to the left appear to do in Fig. 3.

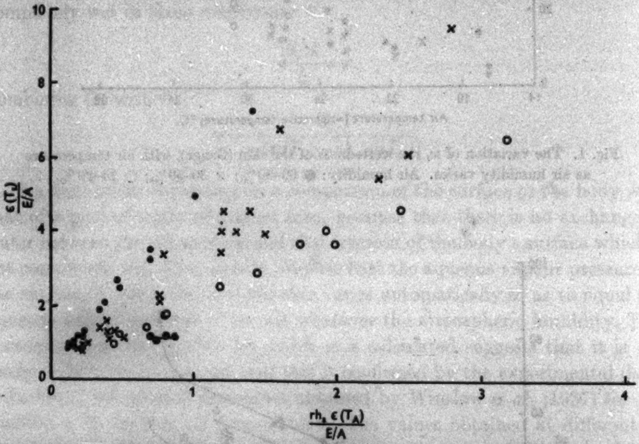

Fig. 3. For explanation of abbreviations see text. ● Points on line of constant rh_s. Aqueous vapour pressure below 10 mm. Hg. Evaporative loss 5–52 kg.cal./sq.m. ○ Points for experiments where aqueous vapour pressure lay between 17 and 22 mm. Hg. Evaporative loss 12–69 kg.cal./sq.m. ⊗ Points for experiments where aqueous vapour pressure exceeded 26 mm. Hg. × All other experiments.

On the assumption that this is a line of constant rh_s, a regression equation may be calculated which gives the value of k without any assumption as to the actual value of rh_s. The equation is

$$(\text{constant } rh_s)\ \frac{\epsilon\,(T_S)}{E/A} = 4{\cdot}95\left(\frac{rh_a\,\epsilon\,(T_A)}{E/A}\right) + 0{\cdot}134.$$

Therefore
$$k = \frac{1}{0{\cdot}134} = 7{\cdot}5.$$

This value for k may be checked by calculating the regression equation for the straight line which appears to join the points from the experiments, where the vapour pressure of the water in the air lay between 17·2 and 21·2 mm. Hg (Fig. 3). From this equation $k=6{\cdot}6$. The agreement between the two values of k is close, considering that a constant rh_s would not be expected in air with a constant aqueous vapour pressure while evaporative loss varied eight-fold.

For a particular value for k, it is possible to calculate from equation (5) the value of rh_s for each set of experimental data given by Winslow *et al.* (1937). For $k=7{\cdot}5$, the values are given in Fig. 2. At a constant air humidity rh_s increases in a linear manner with the air temperature (see also Fig. 4), and with a constant air temperature rh_s increases with the humidity of the air (as would be expected from the way in which rh_s has been calculated). Comparison of Figs. 1 and 2 shows how the use of the variable rh_s has produced a much clearer picture from the individual sets of data than did w, the wettedness of the skin.

Physiological interpretation of rh_s.

The physiological interpretation of an increase in w, the wettedness of the skin, as the humidity of the air increases, is that the increase in w is due to an increased secretion of sweat without the extra sweat adding to the evaporative loss or running off the skin. The interpretation in terms of rh_s is entirely in physical terms and does not invoke any such nicely adjusted physiological process.

It is now usually considered that insensible perspiration depends on a diffusion of water through the skin, and not on any secretory process. The stratum corneum of the skin is the 'waterproof' layer of the skin (Winsor & Burch, 1944), and the rate of diffusion depends on the way in which the epidermis is keratinized (Rothman & Felsher, 1944), and on the removal of water vapour by the environment (Whitehouse, Hancock & Haldane, 1932; Pinson, 1942; Burch & Winsor, 1944). The variation in rh_s with relative humidity and temperature of the air at temperatures below those at which sweating occurs, is consistent with this view of the physical regulation of insensible perspiration. With constant convection and air temperature, an increase in air humidity will temporarily decrease the loss of water vapour from the skin until the continuing diffusion of water from the tissue fluid beneath the skin raises the skin humidity, and hence the evaporative loss of water to the point where this once more equals the rate of water transport by diffusion through the skin.

The experimental data support this view. The consequences of decreased evaporation from the skin, due to increased air humidity, should be a rise in skin temperature, a decrease in the temperature gradient through the skin, and an increase in the temperature difference between skin and air. All these effects occurred (Table 1, cols. 7, 8 and 10), though the changes were small.

The apparent fall in body temperature with increased air humidity (Table 1, col. 9), possibly a chance observation, will have decreased the absolute change in skin temperature.

TABLE 1. The effect of air humidity on the temperature gradients between body, skin and air

Experimental group (1)	Range of T_A (2)	Mean T_A (3)	Mean rh_a (4)	Mean rh_s (5)	Mean (T_B-T_A) (6)	Mean (T_B-T_S) (7)	Mean (T_S-T_A) (8)	Mean T_B (9)	Mean T_S (10)
Low humidity of the air (less than 40%)									
II	24·3–24·4	24·3	33	25	12·7	5·4	7·3	37·1	31·7
III	27·5–30·6	29·1	24	24	8·0	3·8	4·2	37·1	33·3
IV	33·1–34·4	33·9	29	34	3·2	1·8	1·3	36·9	35·2
High humidity of the air (greater than 40%)									
II	21·6–25·3	23·8	64	42	12·8	4·4	8·5	36·7	32·3
III	27·6–31·1	29·4	60	51	7·5	2·5	5·1	36·9	34·4
IV	33·0–34·9	33·9	63	70	3·0	1·5	1·5	36·9	35·4

All the means except those in col. (5) have been recalculated from the original data given in Table 1 of Winslow *et al.* (1937). T_A, T_B and T_S are the temperatures in ° C. of the air, the rectum, and the skin respectively. rh_a and rh_s are the relative humidities of the air and the skin respectively.

The relative humidity of the skin, rh_s, may be taken as a measure of the water content of the surface layer of the skin, expressed as a percentage of the maximum when the skin surface is covered by a homogeneous film of water. It is thus closely analogous to the relative humidity of the air, and measures the partial pressure of water vapour at the skin surface as a fraction of the maximum 'saturation' value at the skin temperature. The skin surface, however, is not homogeneous like the air, and rh_s can only be an average figure. Further, there is no necessity for rh_s to be uniform over the different parts of the body, as has been assumed in the calculations.

The relative humidity and thermal conductivity of the skin

There is a close relation between the mean values of rh_s and of the thermal conductivity of the skin when the experiments are grouped according to the temperature and humidity of the air (Table 2, cols. 2–4). The relation is much closer than that between w and conductivity (Winslow *et al.* 1937). Thermal conductivity of the skin $=\dfrac{E+R+C}{A\,(T_B-T_S)}$ (Table 2), and the greater part of the differences in conductivity in the different experimental groups is related to the difference in skin-temperature gradients which have already been shown to vary inversely with air humidity (Table 1, col. 7, and Table 2, col. 6). There is also a systematic difference according to air humidity in the numerator of the ratio (Table 2, col. 7), which is due to a systematic difference in its radiation and convection components (Table 2, col. 10). Skin evaporation (E_S) is invariant with air humidity. Evaporative loss from the respiratory passages (E_R) does vary, but the equivalent differences in heat exchange are small.

Heat exchange by radiation and convection was calculated from the temperature differences between skin and air which have already been shown to vary with humidity (Table 1, col. 8), and this accounts for the increase in the numerator of the conductivity equation.

TABLE 2. Analysis of the effect of air humidity on the thermal conductivity of the skin

Experimental group (1)	Mean rh_s (2)	$\frac{\text{Col. (4)}}{\text{Col. (2)}}$ (3)	Mean conductivity (4)	Col. (6) × Col. (7) (5)	Mean $\frac{1}{T_B - T_S}$ (6)	Mean $\frac{E+R+C}{A}$ (7)	Mean $\frac{E_S}{A}$ (8)	Mean $\frac{E_R}{A}$ (9)	Mean $\frac{R+C}{A}$ (10)
			Low humidity (less than 40 %)						
II	25	0·61	15·3	15	0·19	77	14	5	64
III	24	0·61	15·0	15	0·28	55	19	5	36
IV	34	0·88	29·5*	30	0·60	51	39	5	12
			High humidity (more than 40 %)						
II	42	0·46	19·5	19	0·23	86	12	4	75
III	51	0·65	32·9	33	0·50	66	20	3	45
IV	70	0·63	47·6†	44	0·86	51†	36†	2	15

* Recalculated from original data.

† Recalculated using measured *E*—see footnote to Table 1 of Winslow *et al.* (1937).

E, *R* and *C* are the heat exchanges in kg.cal./hr. due to evaporation, radiation, and convection respectively; E_S and E_R are the evaporative losses, expressed in the same units, from skin and respiratory passages respectively; *A* is the Du Bois area of the body in sq.m.

Hence the effect of an increased air humidity on the thermal conductivity of the skin seems to be entirely due to the effect of air humidity on skin temperature discussed above, and the parallelism between the relative humidity and thermal conductivity of the skin seems to be a necessary consequence of the relative impermeability of the skin to the diffusion of water.

It does not seem probable that alterations in skin humidity could affect the specific conductance of tissues deep to the epidermis. It is more likely that alterations in air humidity, and therefore in rh_s, are associated with alterations in the distribution and flow of blood in the vessels of the skin as pointed out by Winslow *et al.* (1937). This deduction may have a clinical application, since the humidity of the air has popularly been supposed to have effects on disease for which there seemed to be no possible mechanism.

The wet area of the skin

A physical mechanism adequately accounts for variations in rh_s at temperatures below the critical temperature for the onset of sweating, but, when sweating starts, the physiological interpretation of relative humidity of the skin is less clear. The sweat appears at the skin surface in small droplets which must have a relative humidity of 100%. The skin between the droplets of sweat presumably behaves as outlined above. Some abrupt increase in the humidity of the skin might be expected, depending on the proportions of the relatively humid areas of the skin surface and the areas covered by liquid sweat. The points in Fig. 2 for air humidities of 70–80 % are numerous enough for an increasing skin humidity to be appreciated at air temperatures above 34° C.

The additional data given by Gagge (1937) and derived from experiments where air and operative temperature varied independently also serve to illustrate this point. In Fig. 4, against air temperature, are plotted the values of rh_s calculated from experiments selected because the air humidity was constant. For operative temperatures up to 32° C., the values of rh_s bear a linear relation to the air temperature (evaporative loss 8–51 kg.cal./hr.). At higher operative temperatures, the values of rh_s are increased, and the points lie progressively farther from the straight line. This is because of the greater sweat secretion at the higher operative temperatures, as shown by evaporative losses of up to 184 kg.cal./hr.

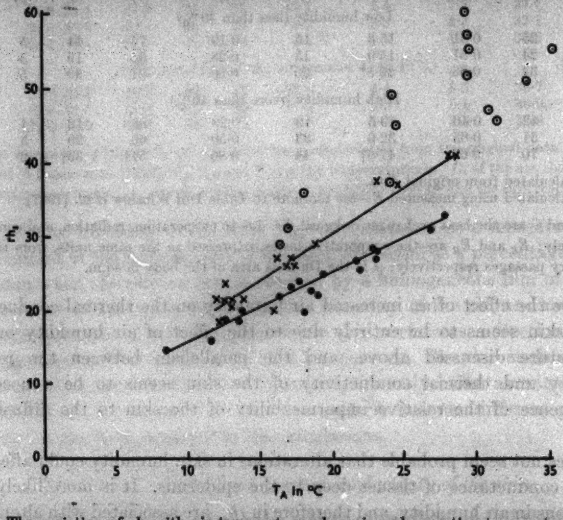

Fig. 4. The variation of rh_s with air temperature when air and operative temperatures differ. For abbreviations see text. T_0=operative temperature. ● T_0 less than 32·5, rh_a 40–44; × T_0 less than 32·5, rh_a 45–52; ⊙ T_0 greater than 32·5, rh_a 40–44.

Let E_w be the evaporative loss from that fraction of the skin surface A_w which is completely covered with liquid sweat. Then

$$E_w/A_w = k\,[\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)].$$

Similarly, let E_{rh} be the evaporative loss from the rest of the skin surface $A - A_w$. Then

$$E_{rh}/A - A_w = k\,[\rho\,\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)],$$

where ρ is the true relative humidity of the skin surface in the areas not completely covered with liquid sweat. Now

$$E/A = k\,[rh_s\,\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)],$$

where E is the measured total evaporative loss from the skin and rh_s is the calculated 'average' relative humidity of the skin.

But $$E = E_w + E_{rh},$$

and k is a constant depending on air movement. Therefore

$$A\,[rh_s\,\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)]$$
$$= A_w\,[\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)] + [A - A_w]\,[\rho\,\epsilon\,(T_S) - rh_a\,\epsilon\,(T_A)],$$

or $$\frac{A_w}{A} = \frac{rh_s - \rho}{1 - \rho} = W, \tag{7}$$

the wet fraction of the skin surface.

It would thus be possible to calculate what fraction of the skin surface was completely covered with sweat if the 'true' rh_s and calculated 'average' rh_s were known. The 'true' rh_s cannot be derived directly from the linear portion of the curve (Fig. 4) without allowing for the effect of skin temperature on rh_s and existing experimental data provide an insufficient basis for this correction. Calculation from the data of Fig. 4, where skin temperature approaches its maximum and this correction is therefore minimal, suggests that 30% of the skin surface may be covered by liquid sweat without this sweat running off the body.

It is interesting that true and calculated rh_s begin to diverge at operative temperatures above 32° C., whereas the critical operative temperature for the onset of sweating, as judged by an increase in the evaporative loss, is 30–31° C. (Winslow *et al.* 1937). There will be an appreciable error in the calculated rh_s only when W is large enough, and this does not seem to occur until the operative temperature is high enough to produce a considerable secretion of sweat.

The limiting environmental conditions for thermal equilibrium

Winslow *et al.* (1937) used formula (4) to deduce the environmental temperatures and humidities at which the upper limit of evaporational cooling was reached. Using formula (5) a similar derivation gives

$$rh_a = \frac{1}{\epsilon\,(T_A)}\left(rh_s\,\epsilon\,(T_S) - \frac{47{\cdot}0 - 8{\cdot}45\,(T_S - T_A)}{k}\right). \tag{8}$$

If T_S at the upper limit is taken to be 35·6° C., as assumed by Winslow *et al.*, and $k = 7{\cdot}5$,

$$rh_a = \frac{1}{\epsilon\,(T_A)}\,(43{\cdot}61 rh_s + 33{\cdot}84 - 1{\cdot}1267 T_A). \tag{9}$$

The values for T_A at different rh_a are given in Table 3.

The maximum temperatures are calculated for two different levels of rh_s. It does not seem likely that rh_s can ever equal 100%, since the greasiness of the skin prevents its complete wetting. Values of up to 88% were obtained experimentally (Fig. 2), and it seems probable that the true maximum 'average' rh_s lies between 80 and 100%, perhaps depending partly on the air humidity. The lower the humidity of the air, the lower the humidity of the areas of skin between the completely wet areas, and hence the lower the 'average' rh_s.

TABLE 3. The environmental limits of thermal equilibrium.

	Max. temp. derived from w 100%			Max. temp. derived from					
				rh_s 80%			rh_s 100%		
Relative humidity of the air	Dry bulb (° C.)	Wet bulb (° C.)	Equiv. basic effective temp. (° F.)	Dry bulb (° C.)	Wet bulb (° C.)	Equiv. basic effective temp. (° F.)	Dry bulb (° C.)	Wet bulb (° C.)	Equiv. basic effective temp. (° F.)
0	45·3	16·6	80	61·0	23·9	—	68·7	26·2	—
13	42·5	21·1	83	50·3	26·4	90	55·0	29·2	94
28	40·0	24·4	84	44·1	28·2	90	48·0	31·0	94
47	37·5	27·8	87	39·1	29·4	89	42·5	32·1	94
70	35·0	30·0	88	34·9	30·3	88	37·9	32·9	93
100	32·5	32·5	90	31·1	31·1	88	33·7	33·7	92

The temperatures from w have been taken from Table 3 of Winslow *et al.* (1937). The equivalent basic effective temperatures have been derived from the nomograms given by Bedford (1946). All these temperatures are calculated for a turbulent air movement of 17 ft./min.

The dry-bulb temperatures for the upper limit of evaporational cooling in dry air are much higher when derived from rh_s than from w; in the first instance, they exceed what is known to be true, in the second they underestimate the maximum temperatures in which man can maintain thermal equilibrium (Robinson, Turrell & Gerking, 1945). In dry air, however, what determines thermal equilibrium is the ability of the body to transport fluid to the skin surface, not the ability of the environment to evaporate moisture at the skin surface. The latter may be greater than the former, and calculations from the evaporative ability of the environment, as in Table 3, would be expected to overestimate the body's capacity to withstand dry heat.

The greatest sweat rate at which it was possible, in a short-term experiment, to replace orally the fluid lost from the skin was of the order of 1·5 l./sq.m./hr. (Ladell, 1945*a*). This rate of fluid transport, however, did not occur under equilibrium conditions. If a sweat rate of 300 ml./sq.m./hr. is taken as a likely upper limit to the secretion of sweat evaporated by acclimatized subjects in conditions of equilibrium, the use of the formula of Winslow *et al.*,

$$E/A = 47{\cdot}0 - 8{\cdot}45\,(T_S - T_A),$$

gives 51° C. (124° F.) as the highest air temperature where thermal equilibrium may be preserved. The maximum humidity corresponding to this dry-bulb temperature would be 12% for $rh_s = 80\%$ and 21% for $rh_s = 100\%$. This agrees with what has been found by experience in desert climates.

The maximum wet-bulb temperatures corresponding to the dry-bulb temperatures are also much nearer the observed limits in experimental observations (Robinson *et al.* 1945) and in the field. The maximum wet-bulb temperatures vary with varying humidity of the air to a much smaller extent than the limiting dry-bulb temperatures, or the limiting wet-bulb temperatures calculated from w and they are in good agreement with the older observations of Haldane.

Brunt (1943) has used the data of Winslow *et al.* (1937) to construct a climatic graph to show the tolerable environmental limits for working, and for existing in comfort and discomfort. Experience of a desert climate has shown that these limits are too low (Ladell, 1945*b*), and observations in a humid climate, where men worked and played games at the upper limits as calculated from w (Table 3), have confirmed that the calculated limits of Brunt are also too low at air humidities of 80% (Horne & Mole, unpublished observations).

The effective temperature-scale was established by equating environments with differing characteristics but which produced the same degree of comfort or discomfort in trained observers. It might be expected, therefore, that at the limits of thermal equilibrium the effective temperature would be constant and independent of the humidity. This was found to be so when these limits were calculated from rh_s (Table 3). Yaglou (cited by Bedford, 1946) has already given 90° F. as the highest effective temperature which can be endured without serious effects by persons at rest or doing light work and it is noteworthy that the values calculated from rh_s and based on experimental data of a completely different kind agree so well with his empirical findings.

DISCUSSION

In the derivation of w, or wettedness of the skin (Gagge, 1937), insensible perspiration and sweat are considered together as one source of evaporative loss of water. Alterations in w were supposed to be due to a rather finely adjusted secretion of sweat. Such a unitary concept is opposed to orthodox physiological teaching, which sharply distinguishes insensible perspiration, governed entirely by the physical factor of diffusion of water through the skin, from sweat, a glandular secretion under physiological control. Analysis of the experimental data on which w, the wettedness of the skin, was founded, suggests that these data fit the orthodox framework more easily. rh_s is governed by physical factors and W depends on a sufficient sweat secretion. By extrapolation this analysis, too, led to a calculation of the environmental limits for thermal equilibrium much nearer the observed limits than when w was used.

Experimental verification of the analysis in terms of rh_s is theoretically possible. Highly humid air at temperatures approximating to skin temperature should prevent the evaporation of water from the skin. If it were possible to keep the walls of the 'partitioning calorimeter' (Winslow, Herrington & Gagge, 1936) at such a low temperature that radiation losses from the body obviated the necessity for sweating, a reduction in evaporation of insensible perspiration could be measured and equation (5) verified at one of its logical extremes.

The relative humidity of the skin varies with air temperature, not with the operative temperature. As explained by Gagge (1940) the humidity of the air is not one of the variables that directly affects the operative temperature. Hence a variable which is a function of the humidity of the air would not be expected to vary regularly with the operative temperature. This is perhaps another reason for discarding w since w does vary with the operative temperature (Gagge, 1937).

The numerical values of w were calculated by assuming that in certain experiments the whole body surface was 'wet' ($w=100\%$), and that the measured evaporative loss then exceeded the true evaporative loss of the body so producing a divergence from linearity in the relation of metabolism, heat storage and loss by evaporation on the one hand, and heat loss (or gain) by radiation and convection on the other. Such an excess of measured over true evaporative loss could only occur if sweat fell on to the floor or wetted the canvas chair on which the experimental subject reclined. Calculation using the data given shows that an area of up to one-quarter of the body's surface must have been wetted in this manner to account for the excess evaporation in the non-linear experiments (Gagge, 1937) and in a further group of experiments at high humidities (Winslow *et al.* 1937). In the light of the evident care with which these experiments in partitional calorimetry were carried out, it does not seem likely that such extensive wetting of surfaces other than the body occurred without being observed. The explanation was only invoked to account for the non-linearity of the results, and the calculation suggests that there may have been some other explanation for part at least of these divergences from linearity.

SUMMARY

1. The experimental data of Winslow, Herrington & Gagge (1937) have been re-analysed in terms of rh_s, the relative humidity of the skin.

2. The relative humidity of the skin depends on the diffusion of water through the skin, on the skin temperature, on the vapour pressure of water in the environment and also on air movement.

3. At a constant air humidity the relative humidity of the skin increases in a linear manner with the air temperature up to a critical operative temperature of about 32° C. Above this temperature, increasing areas of the skin surface are completely covered by sweat and the calculation of rh_s becomes more complex.

4. At a constant air temperature the relative humidity of the skin increases with the humidity of the air.

5. The temperature gradient from body to skin varies inversely and the gradient from skin to air directly with the humidity of the air.

6. The relative humidity of the skin varies closely with the thermal conductivity of the skin.

7. The variable *w*, the wettedness of the skin (Gagge, 1937), has been criticized because it does not seem able to account for the observed fact of the independence of evaporative loss and air humidity as well as the variable rh_s introduced in this paper to replace it, and because its physiological interpretation seems improbable.

8. More accurate conclusions on the upper environmental limits of thermal equilibrium can be reached from the same experimental data by using the variable rh_s instead of *w*.

The author's thanks are due to Prof. W. H. Newton for his criticism and encouragement.

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STIMULATION OF THE SUPRAOPTICOHYPOPHYSIAL TRACT IN THE CONSCIOUS RABBIT WITH CURRENTS OF DIFFERENT WAVE FORM

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(*Received* 29 *September* 1947)

Stimulation of the neurohypophysis in the conscious rabbit (Harris, 1947) has been shown to cause inhibition of a water diuresis, increase of urinary chloride and an increase in activity of the oestrous or oestrogenized uterus. The remote control method of stimulation was used in these experiments, whereby a small secondary coil was buried in a rabbit and an electrode led to some part of the neurohypophysis. Stimulation was performed by holding a large iron-cored primary coil, in series with the a.c. mains, close to the animal's head so that the buried secondary coil was then situated in an electromagnetic field. In the course of further work it became desirable to replace the heavy iron-cored coil (weight 1·6 kg.) with a lighter and less cumbersome coil, and for this purpose it was found essential to use current pulses in the primary, and so in the buried secondary coil, of a different wave form. In view of the results obtained by Hess (1932), and by Harrison, Magoun & Ranson (1938), on the most advantageous wave form for electrical stimulation of the hypothalamus, it was necessary to compare the efficiency of the two different methods in exciting the supraopticohypophysial tract and secretion of the neurohypophysis.

METHODS AND RESULTS

The buried unit and the method of inserting it have been described previously (Harris, 1947).

Circuit 1 (Mains-series-coil stimulator). The primary circuit consisted of the heavy iron-cored coil in series with a variable resistance connected with the commercial a.c. mains (50 cyc./sec., 200 V.). The wave form of the pulses in a primary and secondary coil are given in Fig. 1.

Circuit 2 (Thyratron stimulator). The more convenient form of primary coil (weight 0·2 kg.) and circuit is given in Fig. 2. The wave forms of the pulses produced by this circuit in primary and secondary coils are given in Fig. 3.

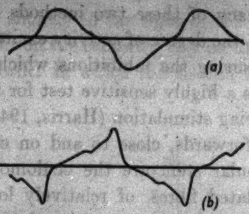

Fig. 1. Tracings of oscilloscope photographs of pulses produced in (*a*) the primary coil, and (*b*) a secondary coil, by the mains-series-coil stimulator. Compare with the wave form of the pulses produced by the thyratron stimulators illustrated in Figs. 3 and 6.

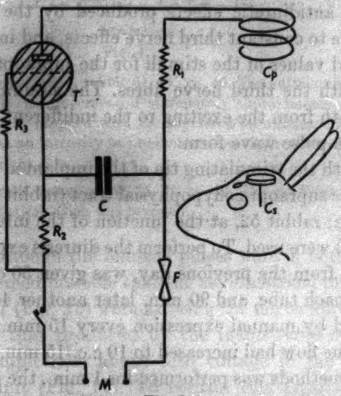

Fig. 2.

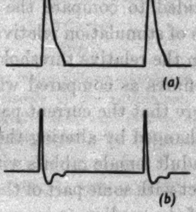

Fig. 3.

Fig. 2. Circuit 2. Thyratron stimulator. *C*, condenser bank 30–40 μF. (condensers suitable for operation in a.c. currents); C_p, three primary coils, each consisting of 140 turns of enamelled copper wire (s.w.g. 22), the dimensions of the coils being—mean diameter 4·5 in., length of winding 0·6 in., and radial depth 0·5 in.; C_s, implanted unit; *F*, fuse; *M*, mains supply 200 V., 50 cyc./sec., a.c.; R_1, resistance 5–10 ohms; R_2, resistance 40 ohms; R_3, resistance 10 k. ohms; *T*, thyratron valve, B.T. 5. The filament and filament transformer of the valve are omitted. In this circuit the value of the resistance R_2 must be kept low to ensure that the condenser *C* is charged to full voltage of the supply. At the instant this is reached, the voltage drop across R_2 due to the condenser current will fall below the critical control grid voltage of the thyratron which will thereupon discharge the condenser into the load. Unfortunately the thyratron will continue to pass a small current after the main discharge until the polarity of the supply reverses. This results in a terminal flattening of the pulse wave (Fig. 3*a*) and a higher mean current, which is a disadvantage particularly when applying prolonged stimuli. For applying short periods of stimulation this circuit and coil are very satisfactory.

Fig. 3. Tracings of oscilloscope photographs of pulses produced in (*a*) the primary coil, and (*b*) a secondary coil, by the thyratron stimulator.

To compare the efficacy of these two methods of stimulating the supraopticohypophysial tract the degree of activity excited in the neurohypophysis was estimated by measuring the inhibitions which such stimuli exerted on a water diuresis. This is a highly sensitive test for the liberation of the antidiuretic hormone following stimulation (Harris, 1947). Use was also made of the fact that running forwards, close to and on either side of, the median eminence and infundibular stem are the oculomotor nerves. These nerves consist of large medullated fibres, of relatively low threshold to electrical excitation. Stimuli strong enough to excite the relatively insensitive, unmyelinated fibres in the hypophysial stalk will at the same time excite the oculomotor nerves and thus produce visible evidence of stimulation (elevation of the upper eyelids, rotation of the eyeballs and enophthalmos). Therefore it was decided to compare the antidiuretic effects produced by the different methods of stimulation relative to constant third nerve effects, and in this way ascertain the relative threshold values of the stimuli for the supraopticohypophysial fibres as compared with the third nerve fibres. The assumption was made here that the current path from the exciting to the indifferent electrode was unchanged by altering the pulse wave form.

Two adult female rabbits with the stimulating tip of the implanted electrode in contact with some part of the supraopticohypophysial tract (rabbit 51, in the region of the median eminence; rabbit 52, at the junction of the infundibular stem and infundibular process) were used. To perform the diuresis experiments the rabbit, which was starved from the previous day, was given 50 c.c. warm water/kg. body weight by stomach tube, and 90 min. later another 40 c.c./kg. The bladder was then emptied by manual expression every 15 min., and the urine measured. When the urine flow had increased to 10 c.c./15 min. or more, stimulation by one of the two methods was performed for 1 min., the effects on the orbital structures being carefully noted. After the antidiuresis so produced was over, and the rate of urine flow rising sharply, stimulation was performed by the other method for the same duration, and at an intensity to produce similar third nerve effects. On a following day, this procedure was repeated, the order of applying the stimuli being reversed. This was thought to be necessary since repeated stimuli of the same intensity, applied to the supraopticohypophysial tract during a water diuresis, produce progressively greater antidiuretic responses (Harris, 1947).

Seven such experiments on the two rabbits have shown little difference in the antidiureses elicited by the two methods of stimulation when these produced the same effects through the third nerve. Fig. 4 shows the effect of the two different stimuli, as applied to the median eminence, upon a water diuresis. Both stimuli were of 50 cyc./sec. frequency and both were applied for 1 min. at such an intensity that moderate elevation of the upper eyelid and enophthalmos was observed on the right side, and no signs on the left (the electrode in this case being situated slightly to the right of the midline). Fig. 5 shows repetition of this experiment on the following day on the same rabbit, the only difference being that the order in which the stimuli were administered was reversed.

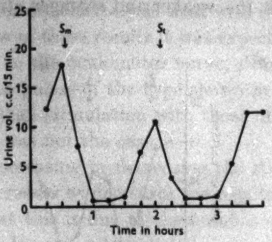

Fig. 4.

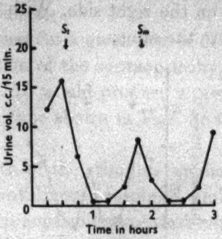

Fig. 5.

Fig. 4. Diuresis curve, rabbit 51, 21. xii. 46. The antidiuresis which follows stimulus S_m (applied with the mains-series-coil stimulator) approximates closely to the antidiuresis following stimulus S_t (applied with the thyratron stimulator, Fig. 2). Both stimuli were applied for 1 min. at an intensity to produce moderate elevation of the upper eyelid and enophthalmos on the right side.

Fig. 5. Diuresis curve, rabbit 51, 22. xii. 46. The conditions were the same as in Fig. 4, but the order of stimulation was reversed.

Now although the induced pulses were in both cases a.c. in type at 50 cyc./sec., it seemed possible that the asymmetrical pulses induced by the thyratron stimulator (Fig. 3*b*) were exciting the nerve fibres at an effective frequency of only half that produced by the mains-series-coil stimulator, which induces pulses symmetrical about zero voltage in the implanted unit (Fig. 1*b*). To control this possibility two identical thyratron pulse circuits (each similar to circuit 2) were constructed and arranged so that identical d.c. pulses, in opposite directions and 180° out of phase, were discharged through a doubly wound primary coil. The wave form of the pulses induced in a secondary coil are shown in Fig. 6. Comparison was then made between the effectiveness of this double-thyratron stimulator and the mains-series-coil stimulator in the manner described above. Again, in five experiments on the two rabbits, no difference could be detected in the responses produced by the two methods. Figs. 7 and 8 show the antidiuretic effects produced by stimulation for 1 min. with the double-thyratron circuit and the mains-series-coil circuit. In the experiment shown in Fig. 7 both stimuli lasted for 1 min. and only excited slight elevation of the upper eyelid and enophthalmos on the right side. On the following

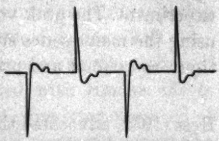

Fig. 6. Tracing of an oscilloscope photograph of pulses produced in a secondary coil by the double-thyratron stimulator.

day the experiment was repeated but the order of applying the stimuli was reversed. By varying the position of the primary coil relative to the animal's head bearing the implanted secondary, the intensity of stimulation was increased till moderate elevation of the upper eyelid and enophthalmos was obtained on the right side. With both the weaker and stronger stimuli the effects were the same.

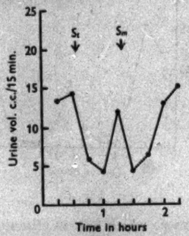

Fig. 7.

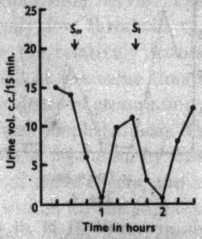

Fig. 8.

Fig. 7. Diuresis curve, rabbit 51, 3. i. 47. The antidiuresis which follows stimulus S_t (applied with the double-thyratron stimulator) approximates closely to the antidiuresis following stimulus S_m (applied with the mains-series-coil stimulator). Both stimuli applied for 1 min. at an intensity to produce slight elevation of the upper eyelid and enophthalmos on the right side.

Fig. 8. Diuresis curve, rabbit 51, 4. i. 47. The conditions were the same as in Fig. 7, but the order of stimulation was reversed.

After completing the experiment shown in Fig. 7, oscilloscope measurements were made of the peak voltages induced in a small secondary coil similar to the implanted coils. The primary coils were suspended over a secondary at distances equal to those between the primary coils and the implanted unit during the experiment. The peak voltages recorded from the secondary coil were 0·97 V. using the mains-series stimulator and primary, and 0·81 V. using the double-thyratron circuit and primary.

DISCUSSION

Hess (1932) advocated the use of direct, damped current pulses for stimulation of the diencephalon, at a frequency of about 8/sec. From the results of experiments on peripheral nerves (ulnar, vagus, sympathetic and depressor nerves) he claimed that the ratio of threshold stimuli for somatic and visceral fibres was nearer unity with damped pulses than with rectangular pulses, and so suggested that there would be less risk of spread to surrounding somatic nerve fibres if damped pulses were used. Harrison, Magoun & Ranson (1938) measured the threshold voltage for stimulation of various parts of the brain stem with induction shocks, rectangular d.c. pulses and modified d.c. pulses. These experiments were performed under anaesthesia, which affected the threshold to a considerable extent in some cases. Their results, however, confirmed Hess's on peripheral nerves, though different conclusions were drawn.

These authors suggested that variation in threshold should be correlated not with fibre type (somatic or visceral) but with fibre size and the phenomenon of accommodation. Also they claimed that it is less dangerous to use induction shocks for prolonged stimulation, as there is less fear of polarization and tissue destruction by electrolysis than with d.c. pulses.

In view of these results it was expected that for a given degree of spread of stimulus to the oculomotor nerve, stimulation of the supraopticohypophysial tract with pulses of the type shown in Fig. 1*b* would produce a greater antidiuresis than stimulation with those of the type shown in Figs. 3*b* or 6. This however was not the case.

Now increasing or decreasing the strength of the stimulus applied within limits increases or decreases the resultant antidiuretic response, and it seems likely that this is due to the number of supraopticohypophysial nerve fibres affected. It cannot be concluded, however, that the different types of stimuli described above, which excite similar antidiuretic responses, are activating equal numbers of supraopticohypophysial nerve fibres. Ignorance of the secretomotor nerve endings in the neurohypophysis makes it impossible to draw conclusions, in terms of nervous excitation, from the magnitude of the antidiuretic response. It is also possible that the discrepancy that seems to exist between these results and those of Hess (1932) and Harrison, Magoun & Ranson (1938) may be explained in terms of the different frequencies of stimulation used. For the present purpose, however, it is important to note that the results of stimulating the neurohypophysis, for periods of 1 min. by the different methods, are strictly comparable for given third nerve effects.

SUMMARY

1. A convenient, simple thyratron pulse circuit and primary coil, for applying short periods of stimulation by the remote control method, is described.

2. In the unanaesthetized rabbit the antidiuretic responses to stimulation of the hypophysial stalk by this method are compared with similar effects elicited by the method previously in use (in which the stimulator consisted of a cumbersome, iron-cored primary coil in series with a resistance across the a.c. mains). The antidiureses produced were found to be similar in magnitude for a given spread of current to the adjacent oculomotor nerve, in spite of the differences in wave form of the pulses induced in the secondary coils.

It gives me pleasure to acknowledge the kindness and encouragement of Prof. H. A. Harris. I am indebted to Mr L. Herbert of the Research Laboratories of the British Thomson-Houston Co. Ltd., Rugby, for advice on the thyratron circuit. The expenses of this research were in part defrayed by a Parliamentary Grant-in-Aid administered by the Royal Society.

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ELECTRICAL STIMULATION OF THE HYPOTHALAMUS AND THE MECHANISM OF NEURAL CONTROL OF THE ADENOHYPOPHYSIS

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(*Received* 29 *September* 1947)

The means whereby the external environment influences the activity of most endocrine glands is unknown. There is no sound evidence that the thyroid, adrenal cortex or gonads possess a secretomotor nerve supply, and it is likely that the trophic hormones of the pituitary gland are the major factors which directly control their activity. The position regarding environmental influence over these glands would be clarified then if a neural or neuro-vascular connexion could be demonstrated between the adenohypophysis (anterior pituitary) and the central nervous system, particularly the hypothalamus.

To investigate the control which the hypothalamus may exert over the secretion of the adenohypophysis a technique for applying prolonged electrical stimulation to these structures in conscious animals has been devised. By this method it is possible to stimulate up to six rabbits simultaneously for several hours daily over periods of weeks or months.

Some workers have implanted electrodes in the cranium and brought leads through the skin. After recovery from the anaesthetic, stimulation was performed by means of the protruding terminals. Disadvantages of this method are that infection may track down the wire, or the wire may break. Another method is to bury a small secondary coil under the scalp, taking leads from this coil to the excitable focus. After the animal has recovered from the preliminary operation the stimulus may be induced through the intact skin from a large primary coil outside the animal. This latter technique, usually termed the remote control method of stimulation, has been used in the present work.

For inducing the stimulus from the primary coil, Fender (1941) and Greig & Ritchie (1944) pass a high frequency current (430 kcyc./sec., Fender; 100 kcyc./sec., Greig & Ritchie) modulated at a lower frequency (60 cyc./sec., Fender; variable, Greig & Ritchie) through the primary coil. The implanted units, which here consist of coils, small rectifiers and condensers are so arranged that the final stimulus consists of direct current pulses of rectangular wave form, at the modulating frequency. The advantage of this method is the ease with which the necessary voltage may be induced in the buried coil. Another method (Loucks, 1934; Chaffee & Light, 1934; Clark & Ward, 1937; and Harris, 1947*a*) is to pass relatively low frequency pulses through the primary coil and use the same frequency pulses in the secondary coil for stimulation. With this technique there is less risk of chemical changes, and consequent damage occurring at the electrode tips since a.c. instead of d.c. pulses are used for stimulation, but the peak current in the primary coil has to be larger. The value of the peak current necessary varies with the dimensions of the two coils, the number of turns in the coils and other factors. Chaffee & Light (1934), who wound the primary coil around the animal's cage, used a special mercury pool vacuum tube to discharge a large condenser bank, and so obtained a peak current in the primary coil of about 1000 amp. This method could be duplicated with the ignitron, though a fairly high-power source of supply for charging the condenser bank is necessary. For the present purpose it was found easier to immobilize the animals and use a smaller primary coil and therefore smaller peak current in the coil.

The following account describes the method used to immobilize the rabbits, and the apparatus for inducing the stimulus. The buried coil, and the technique of implanting it has been described previously (Harris, 1947*a*). The results of stimulation of the hypothalamus, or hypophysis, in exciting the secretion of the gonadotrophic hormone(s), are given.

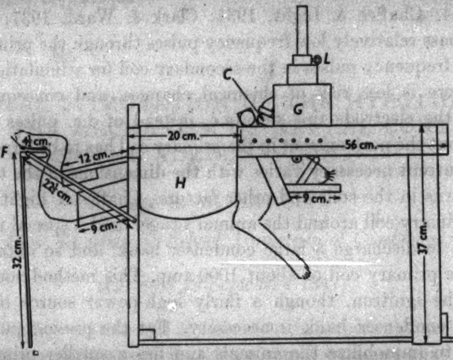
METHOD

The rabbit holder. In order to apply a constant stimulus by means of a primary coil of only a few inches diameter, it is necessary to immobilize the rabbit's head. The holder that was eventually found to be satisfactory is a modification of that described by Laug (1944). Essentially it consists (see Text-figs. 1–5) of two sliding stocks which confine the neck and hips of the animal, a platform support for the lower jaw and head in front of the neck stock, a wide canvas hammock maintaining the chest and abdomen between the two stocks, and another platform support for the thighs behind the hip stock. The forelimbs hang freely between the neck stock and hammock. The head and nose are further stabilized by an arch-shaped nose piece, inclining upwards and forwards from the small platform supporting the lower jaw. Pressure or traction palsies have not been seen. Care has been taken to see that all pressure points on the stock are padded with rubber or felt, and, when inserting the animal, that the hammock extends forwards to give firm support to the thorax. A simple but important addition to Laug's holder is a horizontal flange on which rest the dorsa of the feet. This keeps the hindlimbs well flexed and so obviates any danger of sudden and powerful extensor thrusts, movements by which the animal may fracture the vertebral column.

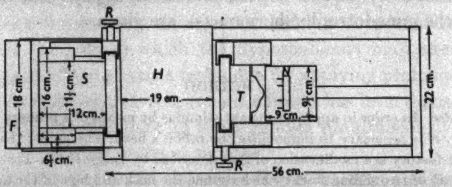
The whole apparatus is built on a rectangular wooden frame with as little metal work as possible. Details and measurements of the holders are given in Text-figs. 1–5.

The primary circuit and coils. In order to be able to stimulate several animals simultaneously, d.c. pulses were passed through a number of primary coils arranged in series in their respective holders.

The thyratron circuit described previously (Harris, 1948) is unsuitable for prolonged periods of stimulation owing to the high mean current. The mean current may be reduced, however, by inserting a half-wave rectifier in the condenser-charging circuit and by controlling the time in the cycle of the condenser discharge. The resulting circuit, used in the present experiments, is shown in



Text-fig. 1. Diagram showing lateral view of a holder with the rabbit in position. × $\frac{1}{10}$. *C*, primary coil, with its carrier, *G*, which slides on the two horizontal supports; *F*, horizontal flange, loosely supporting the hindlimbs in the flexed position, of such dimensions that the feet cannot be withdrawn forwards or sideways; *H*, canvas hammock; *L*, locking screw and collar for adjusting height of primary coil.



Text-fig. 2. Plan of holder. × $\frac{1}{10}$. The primary coil and its carrier have been omitted. *F*, horizontal flange support for the hindlimbs; *H*, canvas hammock; *N*, nose piece on platform support for lower jaw; *R*, ratchet mechanisms for locking sliding pieces of neck and hip stocks; *S*, obliquely placed flange attached to sliding piece of hip stock; *T*, neck stock.

Text-fig. 6 and is similar in principle to that described by Loucks (1934). The frequency of discharge is fixed at that of the mains supply (50 cyc./sec.), but the peak and mean currents, the duration and wave form of the pulses are variable within limits by adjusting the values of R_1, R_2, L and C (see Table 1, and Pl. 2, Fig. 7 *a–c*). Suitable values of R_2 and L have been fixed at 20 ohms and 0·0075 H. respectively. In duplicating this circuit care must be taken that the maximum instantaneous anode current does not exceed the rated value for the valve. For the BT. 5 thyratron this value is 12·5 amp. Table 1 gives the peak current, as measured with an oscilloscope, for different values of the two variables C and R_1. For routine use the following values are taken: C 40 μF. and R_1 0·72 ohm. With this primary circuit and coils, the potentials developed across a secondary coil (of the same dimensions as those implanted in the rabbits) were measured for various intercoil

distances (I.C.D.). The total duration of the biphasic wave was 1·75 msec., and the voltage developed varied between 11·0 V. at I.C.D. 0 cm. to 0·5 V. at 10 cm. (For the wave form of the pulses in the primary and secondary coils see Pl. 2.)

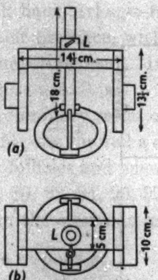


Fig. 3.

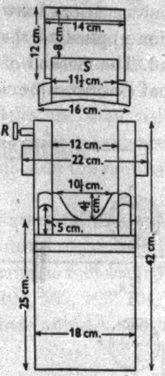


Fig. 4.

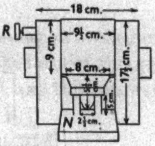


Fig. 5.

Text-fig. 3. Diagram of the primary coil and its carrier. (*a*) Elevation. (*b*) Plan. × $\frac{1}{10}$. *L*, locking screw and collar for adjusting height of coil.

Text-fig. 4. Elevation of hip stock from behind. × $\frac{1}{10}$. *R*, ratchet mechanism for locking sliding piece of stock; *S*, obliquely placed flange attached to sliding piece of stock.

Text-fig. 5. Diagram of neck stock seen from in front. × $\frac{1}{10}$. *R*, ratchet mechanism for locking sliding piece of stock; *N*, nose piece on platform support for lower jaw.

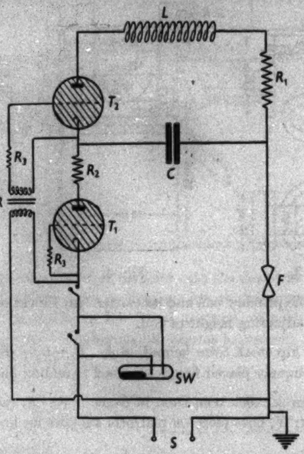
TABLE 1. The peak current through the primary coils, and pulse duration, with R_2 20 ohms, and L 0·0075 H. The mean current varies between 0·5–1·3 amp.

C μF.	R_1 ohm	Peak current in amp.	Duration of pulse in msec.
40	0·18	13·6	1·75
40	0·72	12·4	1·75
40	2·88	11·3	—
40	5·04	10·5	—
40	7·20	9·6	—
40	9·36	9·1	—
40	11·52	8·5	2·0
60	0·72	13·8	2·25
60	7·20	10·2	2·5
60	11·52	8·8	3·0
80	0·72	14·4	2·5
80	7·20	10·5	3·0
80	11·52	8·8	4·0

RESULTS

The quickest and most convenient response known at the present time for testing anterior hypophysial stimulation is the ovarian response (viz. ovulation) in the rabbit. It has been shown previously that hypothalamic or direct hypophysial stimulation for 1 hr. (Harris, 1937), or stimulation of a region above and anterior to the optic chiasma for 30 min. (Haterius & Derbyshire, 1937) in the *anaesthetized* rabbit, was followed by ovulation.

During the past six years, observations have been made on the ovaries after varying periods of stimulation applied to the hypothalamus or to different regions of the pituitary gland. The method of stimulation used in these preliminary observations was that described previously (Harris, 1947*a*), in which



Text-fig. 6. The primary circuit. The condenser bank C is charged in one half of the mains cycle. In the subsequent half-cycle the grid of T_2 becomes positive to the cathode and the condenser C is suddenly discharged through T_2, the six primary coils L, and the small resistance R_1. C, condenser bank 40 μF. (condensers suitable for operation in a.c.); F, 4·5 amp. fuse; L, six primary coils in series, total inductance about 0·0075 H. (each coil consists of 120 turns of copper wire, s.w.g. 18, and has a mean diameter 3·3 in., length 0·8 in., and a radial depth 0·8 in.); R_1 resistance 0·72 ohm; R_2 resistance 20 ohms; R_3 resistance 10 k.ohms; S, mains supply 50 cyc./sec., 200 V.; SW, mercury switch for intermittent stimulation, rocked on a rotating cog-wheel; T_1, T_2, thyratron valves BT. 5; TR, step-down transformer 2 : 1. The filaments and filament transformers of the valves are omitted.

unipolar stimulation was applied by the remote control technique in the *unanaesthetized* animal. The spread of stimulus, using this method, is not more than 0·5 mm. so far as the fibres of the supraopticohypophysial tract are concerned, and this estimate is probably valid for similar unmyelinated fibres in the hypothalamus. The periods of stimulation varied from 1 min. to 1 hr., but in the majority of animals consisted of periods of 5 min. applied daily for several days. In all these experiments the intensity of the stimulus was adjusted by varying the position of the primary coil relative to the animal's head, and standardized by observing the visible effects produced on the orbital structures. These effects are due to spread of current to the adjacent oculomotor nerve which has a low threshold to electrical stimulation. The site of the stimulating tip of the electrode was located, post-mortem, by serially sectioning a block of tissue containing hypothalamus, pituitary gland and surrounding structures. The ovarian response was estimated by naked-eye inspection of the ovaries either at laparotomy or after death. In view of the fact that a few small, black haemorrhagic follicles are often visible in the ovaries of normal animals, their presence, without further evidence of gonadal stimulation, was taken as a negative result. In all cases the rabbits had been isolated for several months before use.

In the preliminary series of sixteen rabbits, stimulation evoked ovulation in two (71, 105); one showed a doubtful response in the form of abnormally large, semi-cystic follicles and haemorrhagic follicles (83), and thirteen were negative (63, 81, 84, 86, 93, 94, 96, 99, 101, 102, 103, 104, 106).

The tip of the electrode in these animals was situated:

(*a*) Positive responses:

71—in the right fornix as it sweeps posteriorly past the paraventricular nucleus.

105—slightly to the left of the midline, in the posterior part of the tuber cinereum.

(*b*) Doubtful response:

83—in the supraopticohypophysial tract at the junction of the median eminence and infundibular stem, and partly in the pars distalis and tuberalis.

(*c*) Negative responses:

Hypothalamus:

63—1 mm. below the left paraventricular nucleus.

99—in the lateral wall of the tuber cinereum.

101—in the posterior wall of the tuber cinereum, midline.

103—in the postero-lateral part of the tuber cinereum.

Neurohypophysis:

86—in the supraopticohypophysial tract at the junction of the median eminence and infundibular stem, and partly in the pars distalis.

106—in contact with the infundibular stem, and in the pars distalis.

81—in the infundibular process.

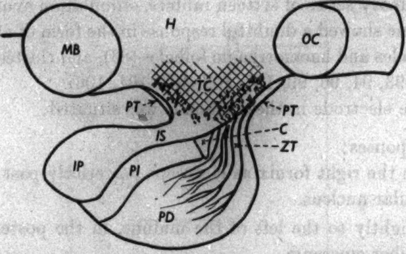
Adenohypophysis:

84, 94, 96, 102, 104—in the pars distalis.

93—in the pars intermedia.

From these rather unexpected results it was thought that the period of stimulation was perhaps insufficient to elicit a full response in many cases, and it was decided to investigate the effects of stimuli of varying duration in more detail.

In the final series, stimulation was applied to seventeen rabbits for periods varying from 1 min. to 7½ hr. using the clamps and thyratron circuit described above. Two or three experiments were performed on each animal. In the first experiment, the period of stimulation chosen was usually 1–3 hr., followed by laparotomy 48 hr. later. After a month, the experiment was repeated with an increased or decreased duration of stimulus according to the previous response. In most cases a third experiment was performed, the animal then being killed.



Text-fig. 7. Diagram of a sagittal section through the hypothalamus and pituitary gland of a rabbit. *C*, connective tissue trabeculae; *H*, hypothalamus; *IP*, infundibular process; *IS*, infundibular stem; *MB*, mamillary body; *OC*, optic chiasma; *PD*, pars distalis; *PI*, pars intermedia; *PT*, pars tuberalis; *TC*, tuber cinereum; *ZT*, zona tuberalis. The area cross-hatched represents the region in which electrical excitation was followed by ovulation. The diagram also illustrates the hypophysial portal vessels, starting as a multitude of sinusoidal loops in the tuber cinereum, uniting to form the wide trunks of the portal vessels in the zona tuberalis, which pass caudo-ventrally and break up into the sinusoids of the pars distalis.

This repetition of the experiment largely eliminates the difficulty of deciding, on a particular occasion, whether a given animal is in full oestrus or not. Gradation of the ovarian response was given by the degree of follicular rupture and the period of stimulation necessary to elicit a given ovarian response.

The results of these experiments are summarized in Table 2, and depicted in Text-fig. 7. It may be seen that out of seventeen rabbits, eight gave the ovulatory response to stimulation, and nine were negative. The electrode was situated in the eight positive cases in some part of the tuber cinereum, and in the nine negative in some part of the adenohypophysis. The duration of the stimulus necessary to evoke full ovulation seemed to vary considerably, and some of the rabbits showed an increasing ovarian response with increasing periods of stimulation. This point will be referred to again later. In the case of rabbit 118, a complete ovulatory response occurred after stimulation of the anterior region of the tuber cinereum for only 3 min., a time period approaching that of the natural stimulus to ovulation in the rabbit, viz. coitus.

TABLE 2

All periods of stimulation were administered 1 min. on : 1 min. off. The figures given above for the duration of stimulation give the actual period of applying the stimuli, viz. '3 min.' represents three periods of 1 min. spread over 5 min. The longer periods were again subdivided, viz. '7½ hr. over 5 days' represents 1 min. on : 1 min. off, for 1 hr., three times a day, for 5 days. The ovarian response is represented: + + +, full complement of ruptured follicles; + +, some ruptured follicles together with haemorrhagic and cystic follicles; +, haemorrhagic and cystic follicles; –, only normal ripe follicles.

	Periods of stimulation							
Rabbit	7½ hr. over 5 days	2–3 hr. over 7 days	2½–3 hr. over 3 days	1–1½ hr. over 1–3 days	10–12 min.	3 min.	1 min.	Electrode site
107	.	.	.	+ + +	.	.	.	Posterior part of tuber cinereum*
115	.	.	+ + +	.	+ +	+	.	Posterior part of tuber cinereum*
116	.	.	+ + +	.	+	–	.	Low in anterior part of tuber cinereum*
118	.	.	+ + +	.	+ + +	+ + +	.	High in anterior part of tuber cinereum*
119	.	.	.	+ +	–	.	.	Anterior part of tuber cinereum
121	+ +	.	–	.	–	.	.	Low in posterior part of tuber cinereum and in infundibular stem
124	.	.	.	.	+ + +	+ +	–	Low in posterior part of tuber cinereum and in infundibular stem
126	.	.	.	+ + +	.	–	.	In right side of anterior part of tuber cinereum
108	.	–	.	–	–	.	.	Pars distalis, just posterior to zona tuberalis
109	.	–	.	.	–	.	.	Pars distalis*
110	–	.	–	–	.	.	.	Zona tuberalis
111	–	.	–	.	–	.	.	Zona tuberalis
120	–	.	.	–	.	.	.	In contact with right side of infundibular stem, and in pars distalis
123	–	.	.	–	–	.	.	Zona tuberalis
127	–	.	.	–	.	.	.	In junction of infundibular stem and infundibular process, and in pars intermedia*
128	.	.	.	–	–	.	.	Pars distalis
129	–	.	.	.	–	.	.	Pars distalis

* See Plate 1, figs. 1–6.

DISCUSSION

The remote control method of stimulation has manifold advantages. It is simple and economical and permits (1) stimulation of the nervous system without anaesthesia, (2) stimulation without simultaneous trauma, (3) repetition of the experiment in the same animal and (4) stimulation of groups of animals over periods of weeks or months.

During the course of this work Markee, Sawyer & Hollinshead (1946) published similar findings on the ovulatory response in the rabbit. Working with animals anaesthetized with ether, they stimulated the pituitary by a pharyngeal or temporal approach, and the hypothalamus through the superior orbital fissure. They found that electrical stimulation of the pituitary gland does not result in ovulation, unless there are signs of spread of the stimulus, whereas stimulation of the hypothalamus at a lower voltage resulted in ovulation in three out of four animals. They infer that the hypothalamus does not exert a direct neural control over the anterior pituitary, but '...a long humoral link forms a part of the hypothalamico-anterior hypophyseal connexion'.

In this present study it has been shown that stimulation of the tuber cinereum is followed by ovulation in the rabbit, whereas direct electrical stimulation of the pars distalis (including the zona tuberalis), pars intermedia or infundibular stem of the pituitary gland with a stimulus of the same strength applied for a much longer period is ineffective. It is thought that previous results (Harris, 1937) may be explained by the fact that the animals were anaesthetized. The most active focus, in the present series of animals, was in the anterior wall of the tuber cinereum just above the anterior part of the median eminence. The stimulus used was of a highly localized nature. For the unmyelinated fibres of the supraopticohypophysial tract the current spread is only effective for distances up to 0·5 mm., and this figure probably holds good for similar fibres in the hypothalamus. It has been shown, for example (Harris, 1947*a*), that, with the electrode tip in the pars intermedia, a maximal response cannot be obtained from the closely adjacent infundibular process. Markee, Sawyer & Hollinshead (1946) obtained ovulation after bipolar stimulation of the hypothalamus, the stimulus spread being evidenced by tremor of the head, neck and fore-limbs. Stimuli strong enough to produce these signs of spread can also be induced by the remote control method, but it is necessary to use anaesthetized animals in applying stimuli of this strength. Much weaker stimuli sufficed in the present work.

The fact that stimulation of the tuber cinereum results in the ovulatory response in the rabbit, whereas direct pituitary stimulation does not, suggests that the anterior pituitary gland lacks a secretomotor nerve supply (cf. electrical stimulation of the denervated adrenal medulla, Cannon & Rosenbleuth, 1937). In this respect it is of particular interest to note that stimulation of the infundibular stem for periods of 7½ hr. in rabbits 120, 127, did not result in ovulation. This argues strongly against a neural pathway passing via the infundibular stem to the anterior pituitary. It is well established, however, that the secretion of the anterior pituitary is the immediate precursor of follicular rupture. The simplest explanation appears to be that the hypothalamus influences the activity of the adenohypophysis by a humoral mechanism. This suggestion has been made, as a tentative alternative to the possibility of a direct nerve supply, for many years (Harris, 1937; Hinsey, 1937; Brooks, 1938; Taubenhaus & Soskin, 1941; and others). From the anatomy of the region, and for other reasons, it seems likely that nervous stimuli cause a humoral transmitter to be liberated into the sinusoidal capillaries of the tuber cinereum (see Text-fig. 7), and transmitted via the trunks of the portal vessels, ventrally into the sinusoids of the anterior pituitary. The anatomy of the structures concerned with, and the experimental evidence relating to, this view have been considered in detail (Harris, 1944; Green & Harris, 1947). The suggestion has also been made (Harris, 1947 *c*) that the significance of the pars tuberalis of the pituitary lies in the fact that in all vertebrates from amphibians to man it establishes contact with the infundibular region of the hypothalamus, thus affording the bed for a vascular pathway between the hypothalamus and pars distalis. If the hypophysial portal vessels form the functional link between the nervous system on the one hand, and the adenohypophysis, and so the thyroid, adrenal cortex and gonads, on the other, it might be suggested that structures of such basic importance would be widely distributed throughout the vertebrates. In all forms examined, from amphibians upwards, the pars tuberalis arises from two specialized lateral lobes of Rathke's pouch, and migrates upwards to establish contact with the base of the brain. Similarly, in all forms so far examined, the pars tuberalis bears a constant relation to the hypophysial portal vessels, in that the upper capillaries in the tuber cinereum arise from the plexus of vessels situated in this part of the gland. Even in the pituitary of the porpoise, which possesses a thick dural septum between the infundibular process and the pars distalis (Geiling, Vos & Oldham, 1940), the pars tuberalis passes upwards anterior to the septum, and contains a highly developed system of portal vessels continuous with the sinusoidal loops and tufts of the tuber cinereum (Harris, 1947 *b*).

This hypothesis of neurovascular transmission of stimuli from the tuber cinereum to the anterior lobe of the pituitary gland offers a possible explanation for many discrepancies that exist in the literature, particularly those dealing with the effects of sectioning the pituitary stalk. Attention has been focused in the past on the possibility of nerve fibres passing via the stalk to the anterior lobe. If this nervous pathway exists, it would almost certainly not regenerate after stalk section, so that normal pituitary function, following stalk section, has been used as evidence against participation of the stalk in a hypothalamo-hypophysial relationship. However, in view of the possibility of vascular transmission of stimuli down the stalk much of this work should be repeated, and observations made concerning any regeneration of the hypophysial portal vessels. There is some evidence that such regeneration does occur (rabbit, Brooks, 1938; rat, Harris, unpublished).

With regard to the above hypothesis of neurovascular transmission, the time relations of the different parts of the process underlying ovulation in the rabbit are of interest. A complete ovulatory response in the rabbit is obtained after coitus (duration a few minutes), or after electrical stimulation of the tuber cinereum (duration 3 min., rabbit 118). Now according to Fee & Parkes (1929) the pituitary must be left in situ for at least 1 hr. after coitus for ovulation to occur some 10 hr. later. It seems unlikely that nervous stimulation of the cells of the adenohypophysis, lasting a few minutes, would cause liberation of gonadotropic hormone(s) into the blood stream to continue for 1 hr. This time lag, however, is more easily understood if the final stimulus to the cells is humorally transmitted.

It might be suggested that the ovulatory mechanism of the rabbit is a specialization, and that conclusions drawn from evidence concerning this reaction may not be of general validity. In this respect it is of interest that stimulation of the tuber cinereum in a few rabbits, for periods of 1 to 3 hr. daily for a week, results in a glycotropic response (Harris, unpublished).

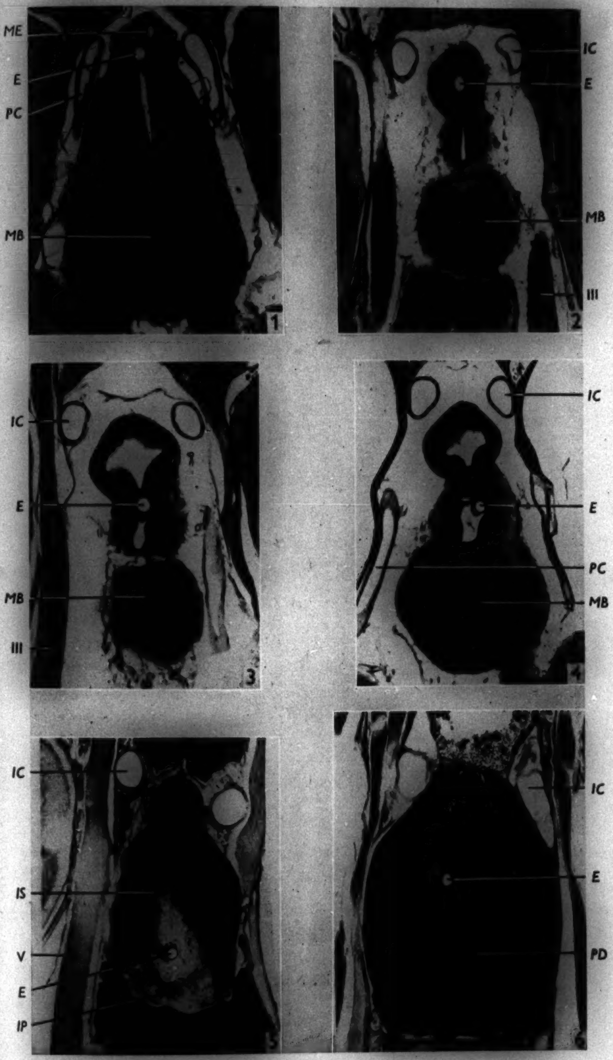
SUMMARY

1. A method is described for stimulating the basal areas of the brain in a group of conscious rabbits simultaneously and for prolonged periods. Up to six rabbits may be immobilized in holders of the stock pattern, and the corresponding primary coils suspended over the animals' heads. The six primary coils are in series and pass 50 cyc./sec., d.c. pulses, produced by a thyratron valve discharge of a condenser bank.

2. By this method it was found that stimulation of the tuber cinereum in the unanaesthetized rabbit, for as short a period as 3 min., may be followed by a full ovulatory response. Direct stimulation of the anterior pituitary, pars intermedia, or infundibular stem for periods up to 7½ hr. was not followed by ovulation.

3. These results are discussed in relation to the possibility of neurovascular transmission of stimuli from the hypothalamus to the anterior lobe of the pituitary via the hypophysial portal vessels.

It gives me great pleasure to acknowledge the kindness and encouragement of Prof. H. A. Harris. I am indebted to Mr E. E. Zepler, Prof. J. Greig, and Mr D. O'Brien for the loan of apparatus, to Mr L. Herbert of the Research Laboratories of the British Thomson-Houston Co. Ltd., Rugby, for advice on the thyratron circuit, to Mr J. A. F. Fozzard for his skilled photography, to Mr L. C. Maltby for his advice and help in building the animal holders, and to Mr R. Smith for his care of the animals and the histological work. The expenses of this research were in part defrayed by a Parliamentary Grant-in-Aid administered by the Royal Society.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Figs. 1–6.

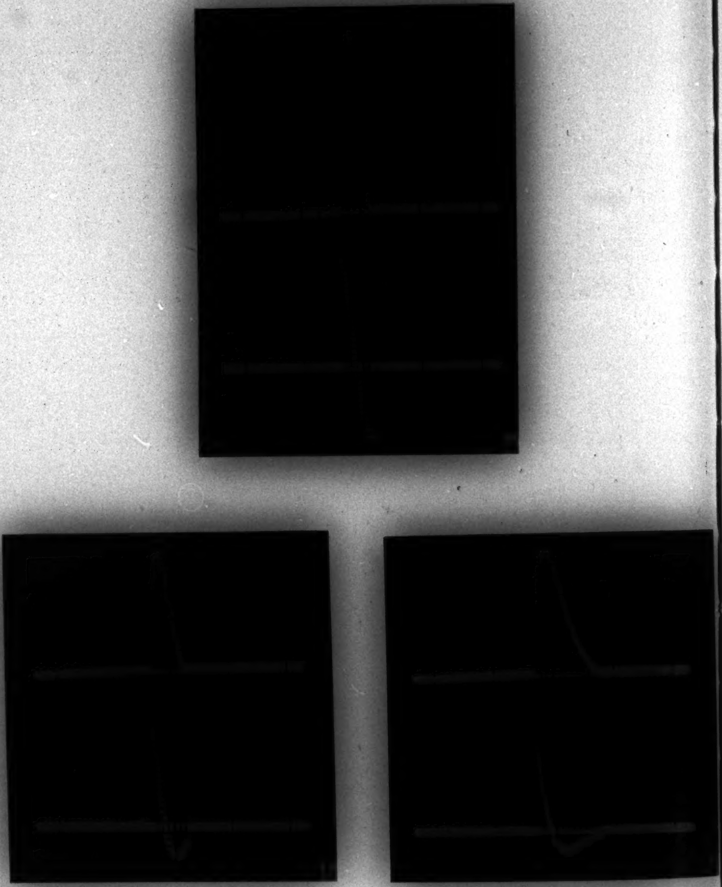

Fig. 7 *a–c*.

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EXPLANATION OF PLATES

PLATE 1

Microphotographs of horizontal sections through the hypothalamus or pituitary gland of some of the experimental animals. In all cases fixation was effected by perfusing the vessels with formalin with the electrode and coil in situ, the electrode site thus appearing as a circular hole in the sections. All sections are 100 μ. thick. (For the effect of stimulation in these cases, see Table 2.) Magnification ×10. *E*, electrode site; *IC*, internal carotid artery; *IP*, infundibular process; *IS*, infundibular stem; *MB*, mamillary bodies; *ME*, median eminence; *PC*, posterior communicating artery; *PD*, pars distalis; *III*, oculomotor nerve; *V*, trigeminal nerve.

Fig. 1. Hypothalamus of rabbit 118. Electrode situated high in the anterior part of the tuber cinereum. The vessels have been injected with india ink solution and some of the upper capillaries of the portal vessels are visible in the median eminence, just anterior to the electrode site.

Fig. 2. Hypothalamus of rabbit 116. Electrode situated in the anterior part of the tuber cinereum.

Fig. 3. Hypothalamus of rabbit 115. Electrode situated in the posterior part of the tuber cinereum.

Fig. 4. Hypothalamus of rabbit 107. Electrode situated in the posterior part of the tuber cinereum.

Fig. 5. Pituitary gland of rabbit 127. Electrode situated in the junction of the infundibular stem and process, and at a lower level penetrating the pars intermedia.

Fig. 6. Pituitary gland of rabbit 109. Electrode situated in the pars distalis.

PLATE 2

Fig. 7*a–c*. Tracings taken with a Cossor double beam oscilloscope. In each case the upper trace is the d.c. pulse through a primary coil, and the lower trace the a.c. pulse through a secondary coil of the same dimensions as those implanted in the rabbits. The primary circuit is shown in Text-fig. 6. The circuit constants were: (*a*) L 0·0075 H., R_2 20 ohms, R_1 0·72 ohm, C 40 μF.; (*b*) L 0·0075 H., R_2 20 ohms, R_1 11·52 ohms, C 40 μF.; (*c*) L 0·0075 H., R_2 20 ohms, R_1 11·52 ohms, C 80 μF.

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THE EXCRETION OF AN ANTIDIURETIC SUBSTANCE BY THE KIDNEY, AFTER ELECTRICAL STIMULATION OF THE NEUROHYPOPHYSIS IN THE UNANAESTHETIZED RABBIT

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(*Received* 29 *September* 1947)

After intravenous injection of posterior pituitary extract the kidney excretes urine containing an antidiuretic substance (Heller & Urban, 1935; Heller, 1937; Hare, Hickey & Hare, 1941). Dehydration of the intact animal produces a similar result, presumably by stimulating the release of the antidiuretic hormone from the posterior pituitary gland (Gilman & Goodman, 1937; Boylston & Ivy, 1938; Ingram, Ladd & Benbow, 1938, 1939; and Hare, Hickey & Hare, 1941). However, Walker (1939) threw doubt on the pituitary origin of the antidiuretic substance found in the urine after water deprivation, and the work of Ham (1941), Ham & Landis (1942) and Schaffer, Cadden & Stander (1941) indicated that the urinary substance was, at least partly, extra-pituitary in origin. The fact that dehydrated hypophysectomized animals (Gilman & Goodman, 1937; and others), or dehydrated animals after interruption of the supraopticohypophysial tracts (Ingram, Ladd & Benbow, 1938, 1939), excrete urine lacking similar antidiuretic activity is strong evidence in favour of a pituitary origin for some of the material.

Investigation of the antidiuretic activity of urine after stimulation of the neurohypophysis was undertaken with two objectives in view; first, to obtain more direct evidence regarding a possible pituitary origin of any such urinary activity, and, secondly, to compare the amount of antidiuretic substance excreted after stimulation of the neurohypophysis for 1 min. with that excreted after the intravenous injection of known doses of posterior pituitary extract. It was hoped that a rough measure could thus be obtained of the amount of antidiuretic hormone liberated from the neurohypophysis after *strong* electrical stimulation. It is impossible to estimate this secretion directly, by assaying the inhibition of a water diuresis in the animal stimulated, for the antidiuresis so produced is outside the range of accurate quantitative assay.

METHODS

Treatment of urine suspected of containing antidiuretic substance. In the first attempts the urine was treated by the method of Gilman & Goodman (1937) by dialysing against running tap water till nearly salt-free. This method was unsuccessful in giving definite or constant results (see Walker, 1939; Schaffer, Cadden & Stander, 1941; Ham & Landis, 1942; and Lipschitz & Stokey, 1947 who report similar findings). The procedure finally adopted was as follows. A rabbit was given 0·5–1 g. sodium dihydrogen phosphate, dissolved in a few c.c. of water, by stomach tube. After 30–60 min. when the urine was acid to litmus, a control sample of urine was taken by manual expression into a clean glass dish, the bladder emptied, and the intravenous injection of posterior pituitary extract, or the stimulation, administered. Since Heller (1937) showed that rabbits excrete antidiuretic substance mainly in the first 15 min. and entirely in the first 30 min. after injection of posterior pituitary extract, the urine to be assayed was collected 30 min. after the injection of the extract, or the stimulation. A few drops of 4% acetic acid were added immediately to the urine samples until the urine was just acid to methyl red (pH 4·7). The samples were finally boiled in a water-bath for 5 min. and filtered. Since posterior pituitary extracts are less stable in alkaline than acid solution, and since the urine of rabbits is normally strongly alkaline, it was felt safer to acidify the urine as excreted by the kidney. This was done by giving NaH_2PO_4 orally.

Injection of posterior pituitary extracts and electrical stimulation of the neurohypophysis. Injections of 'pituitrin' (Parke, Davis & Co.), or 'pituitary (posterior lobe) extract' (Boots Pure Drug Co. Ltd.) were made into the marginal vein of the ear. Stimulation was performed in conscious rabbits by the remote control method (Harris, 1947, 1948*a*).

Assay of urine samples for antidiuretic activity. Walker's (1939) method of assay of antidiuretic activity was used. Normal rabbits were given 50 c.c. warm water/kg. body weight, by stomach tube, and 90 min. later a second dose of 40 c.c./kg. by the same route. The resultant diuresis was estimated by collecting urine samples every 15 min., the bladder being emptied by manual expression. When the urine flow had increased to 8–10 c.c./15 min., the specimen to be assayed (or a known dose of posterior pituitary extract) was injected into the marginal vein of the ear. It was found important to make the injection of acidified urine slowly, and into the vein near the base of the ear where the vein is larger and the blood flow more copious. Injection into the vein towards the tip of the ear often seemed to cause the animal discomfort, which may itself interfere with the course of a water diuresis. As assay animals, Dutch rabbits were used, since they are small and easily handled. It was found advantageous to use the same animals repeatedly as they become accustomed to the procedure, and tend to give better diureses after repetition of the hydration. Additional water was given as required.

RESULTS

The excretion of an antidiuretic substance after the intravenous injection of posterior pituitary extract. To compare the excretion of any antidiuretic substance after intravenous injection of posterior pituitary extract with that following stimulation of the neurohypophysis, two rabbits (51, 52) bearing implanted coil units were used, so that the experiments could be performed on the same animals in sequence. Intravenous injections of posterior pituitary extract were made in doses varying from 2 U. to 50 mU. Accurate assays of the antidiuretic substance excreted in the subsequent half-hourly periods were not made, but detectable amounts of antidiuretic substance were excreted in all cases. The amount of antidiuretic substance excreted seemed to be roughly proportional to the amount of posterior pituitary extract injected (Fig. 1). Control samples of urine, collected before the injection of posterior pituitary extract, possessed no antidiuretic activity.

These results confirm, in the main, those of Heller (1937) who used Burn's rat method of assay.

Assay of the antidiuretic substance excreted in the urine after stimulation of the neurohypophysis. Rabbit 51 (stimulating electrode in contact with the right side of the supraopticohypophysial tract in the median eminence) and rabbit 52 (stimulating electrode in the supraopticohypophysial tract in the junction of the infundibular stem and process) were stimulated strongly for periods of 1 min., and the urine (collected in the ensuing 30 min.) assayed for antidiuretic activity. Similar assays were also performed on rabbit 52 after stimulation for periods of 30 min. and 90 min. (1 min. on : 1 min. off).

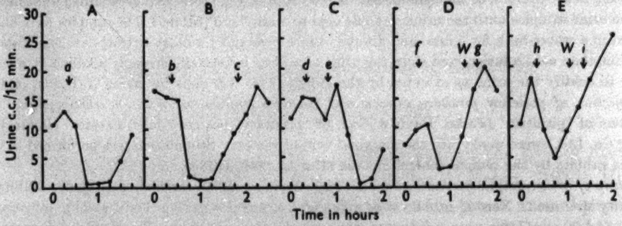

Fig. 1. The excretion of an antidiuretic substance in the urine after intravenous injection of posterior pituitary extract. Diuresis curves of assay rabbit 141 (small Dutch doe). Rabbit 52 was injected intravenously with various doses of posterior pituitary extract, and the urine for assay collected over the subsequent half-hour period. Control specimens of urine collected before injection of extract.

A. *a*, Intravenous injection of $\frac{1}{5}$ × urine excreted after intravenous injection of 2 U.

B. *b*, Intravenous injection of $\frac{1}{2}$ × urine excreted after intravenous injection of 1 U. *c*, Intravenous injection of control urine.

C. *d*, Intravenous injection of control urine. *e*, Intravenous injection of $\frac{1}{2}$ × urine excreted after intravenous injection of 250 mU.

D. *f*, Intravenous injection of urine excreted after intravenous injection of 100 mU. *W*, 60 c.c. water by stomach tube. *g*, Intravenous injection of control urine.

E. *h*, Intravenous injection of urine excreted after intravenous injection of 50 mU. *W*, 50 c.c. water by stomach tube. *i*, Intravenous injection of control urine.

After stimulation of the neurohypophysis for 1 min. very small amounts (less than 1 mU.) of antidiuretic substance were excreted in the urine in the subsequent half-hour. During stimulation of the neurohypophysis for 30 min. the urine excreted contained 2–8 mU. of antidiuretic activity, and similar figures were obtained for urine collected during stimulation for 90 min.

Control procedures. Control samples of urine collected before the injection of posterior pituitary extract, or the stimulation, in no case produced an antidiuresis. It was found that control urine, to which small doses of posterior pituitary extract had been added, exerted an antidiuretic activity comparable to that produced by equal volumes of isotonic sodium chloride to which the same dose of posterior pituitary extract had been added. No detectable antidiuretic substance was excreted during half-hour periods of stimulation in the urine of a rabbit (54) with the electrode situated in the anterior lobe of the pituitary gland, or in the urine of an animal (rabbit 55) that had had the electrode situated in the infundibular stem and pars distalis but in which slight movement of the implanted unit had sectioned the stem with consequent atrophy of the posterior lobe.

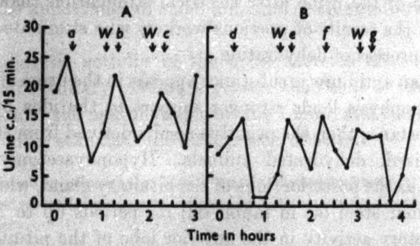

Fig. 2. To compare the excretion of antidiuretic substance in the urine after injection of 50 mU. posterior pituitary extract and stimulation of the neurohypophysis for periods of 1 min. and 30 min. Diuresis curves of assay rabbit 141 (small Dutch doe).

A. Rabbit 52. 28. iv. 47. 2.30 p.m. 50 mU. posterior pituitary extract injected intravenously. 3.00 p.m. Urine collected (specimen *a*). 29. iv. 47. 11.34 a.m. Neurohypophysis stimulated for 1 min. 12.05 p.m. Urine collected (specimen *b*). 29. iv. 47. 4.00 p.m. 50 mU. posterior pituitary extract injected intravenously. 4.30 p.m. Urine collected (specimen *c*). Urine specimens (*a*), (*b*) and (*c*) diluted to 2·5 c.c. with water. These three specimens were injected intravenously into rabbit 141, during a water diuresis, at points *a*, *b* and *c* respectively. At *W*, 80 c.c. water by stomach tube.

B. Rabbits 51 and 52. 13. iv. 47. 10.10 a.m. 50 mU. posterior pituitary extract injected intravenously. 10.40 a.m. Urine collected (specimens *e*, rabbit 52; and *f*, rabbit 51). 14. iv. 47. 12.30–1.00 p.m. Neurohypophysial stimulation. 1.00 p.m. Urine collected (specimens *d*, rabbit 52; and *g*, rabbit 51). Specimens (*d*) and (*e*) diluted to 1·6 c.c. and (*f*) and (*g*) to 2·0 c.c. with water. These four specimens were injected intravenously into rabbit 141, during a water diuresis, at points *d*, *e*, *f* and *g* respectively. At *W*, 50 c.c. water by stomach tube.

Comparison between the amounts of antidiuretic substance excreted after stimulation of the neurohypophysis and injection of posterior pituitary extract. The procedure adopted for these experiments was as follows. A rabbit (51 or 52) was injected intravenously with 50 or 100 mU. posterior pituitary extract, the urine collected, acidified and stored in the refrigerator overnight. On the following morning, the urine was collected from the same rabbit after stimulation, and the antidiuretic activity of the different samples compared on an assay rabbit. By this means it was shown that the intravenous injection of 50 mU. of posterior pituitary extract resulted in the excretion of more antidiuretic substance than followed stimulation of the neurohypophysis for 1 min., but less than followed stimulation (1 min. on : 1 min. off) over a period of 30 min. (Fig. 2).

DISCUSSION

These results confirm most previous work on the excretion of an antidiuretic substance in the urine after intravenous injection of posterior pituitary extract. Stimulation of the neurohypophysis results in the appearance of small amounts of antidiuretic substance in the urine. The assays demonstrated less antidiuretic activity of the urine after electrical stimulation than was expected from a study of the results of previous workers who stimulated the gland indirectly by the process of dehydration.

The fact that an antidiuretic substance appears in the urine after stimulation of the neurohypophysis lends stronger support to the idea of the pituitary origin of the substance than the negative results derived from studies made on hypophysectomized, dehydrated animals. Hypophysectomy removes the anterior, as well as the posterior, lobe of the pituitary gland, whilst stimulation of the infundibular stem (as in rabbit 52) for periods up to 7½ hr. does not excite any secretory activity in the anterior lobe of the pituitary (as judged from the ovaries, Harris, 1948*a*). It is possible, however, that dehydration causes the appearance in the urine of antidiuretic substances of both pituitary and extra-pituitary origin. Ham (1941) and Ham & Landis (1942) claim that the pituitary antidiuretic hormone is lost after dialysis in a cellophane membrane (see also Heller, 1937), whereas the antidiuretic substance found in the urine, which they believe is extra-pituitary in origin, does not pass through cellophane. The view that dehydration causes the appearance in the urine of both types of substance, with dialysing properties as described by Ham (1941) and Ham & Landis (1942), would provide a possible explanation of two difficulties. First, direct stimulation of the neurohypophysis caused the excretion of, what seemed on the results of previous work, unexpectedly small amounts of antidiuretic substance. Calculations from the figures of Gilman & Goodman (1937) indicate that dehydration produces 1–2 mU. antidiuretic substance in the urine of a rat in 30 min., whereas, in the present work, stimulation of the neurohypophysis produced 2–8 mU. antidiuretic substance in the urine of a rabbit formed during the 30 min. stimulation. Secondly, dialysis in cellophane did not result in the loss of the antidiuretic activity of the urine in the work of Gilman & Goodman, though this was the case in the present experiments. Alternative explanations, such as a species difference, or dehydration being a more effective stimulus to the neurohypophysis than electrical stimulation, are also possible.

Stimulation of the neurohypophysis for 1 min. results in the appearance in the urine of slightly less antidiuretic substance than follows the intravenous injection of 50 mU. posterior pituitary extract. On the assumption that the natural hormone and the extract behave similarly in the blood stream and during excretion by the kidney, this would indicate that slightly less than 50 mU. of antidiuretic hormone are liberated from the gland by such stimulation. For a discussion of this subject see Harris (1948*b*).

SUMMARY

1. Intravenous injection of posterior pituitary extract in the rabbit causes the appearance of an antidiuretic substance in the urine.

2. Direct electrical stimulation of the neurohypophysis in the conscious rabbit causes the appearance of small amounts of an antidiuretic substance in the urine.

3. The antidiuretic activity of the urine excreted after the injection of 50 mU. posterior pituitary extract is greater than that of the urine excreted after stimulation of the neurohypophysis for 1 min., and less than that of the urine excreted after stimulation over a period of 30 min.

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FURTHER EVIDENCE REGARDING THE ENDOCRINE STATUS OF THE NEUROHYPOPHYSIS

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It has been shown that lesions of the neurohypophysis or its nerve supply (the supraopticohypophysial tract) result in a state of diabetes insipidus (Fisher, Ingram & Ranson, 1938), and possibly disturbances of the labour mechanism (Fisher, Magoun & Ranson, 1938; Dey, Fisher & Ranson, 1941), and that stimulation of the neurohypophysis or its nerve supply results in inhibition of a water diuresis (Harris, 1947) and contraction of the oestrous uterus (Harris, 1947) or post partum uterus (Haterius & Ferguson, 1938; Ferguson, 1941). The final proof that the actions of posterior pituitary extracts represent true endocrine functions of the gland rests on the demonstration that secretion of the gland, with these reactions as a consequence, occurs as a result of 'natural' stimuli. This has only been established for the antidiuretic hormone (Rydin & Verney, 1938, and see O'Connor, 1947). There is little evidence that the increases in intestinal motility and blood pressure that follow injection of post-pituitary extract represent the actions of any pituitary hormone(s).

In this paper are described the effects on the intestinal motility and blood pressure of stimulating the supraopticohypophysial tract in the unanaesthetized rabbit.

THE NEUROHYPOPHYSIS AND INTESTINAL PERISTALSIS

Bell (1909), Gaddum (1928) and Melville & Stehle (1934) have shown that the intestine of the rabbit is stimulated to increased activity by unfractionated posterior pituitary extracts, or by the pressor fraction. The colon appears to be the most sensitive part of the intestinal tract to the action of such extracts, and the ileum is more sensitive than the jejunum. These results are, however, mainly of pharmacological interest and offer no evidence that the neurohypophysis plays any physiological role in the control of intestinal movements. Suggestive evidence that the neurohypophysis does affect intestinal motility may be found in the work of Wang, Clark, Dey & Ranson (1940) who reported an increase in gastro-intestinal motility in cats under light ether or chloralose anaesthesia after stimulation of the hypothalamus, anterior to the infundibular region. This response had the characteristics of one which was humorally excited (i.e. long latent period, persistence for several minutes after cessation of the stimulus) but was seen in slight form in only four out of eleven cats after spinal cord section. For this reason, the authors were unwilling to emphasize the possible participation of the neurohypophysis in the reaction.

Methods

The remote control method of stimulation in the conscious rabbit was used. The buried unit and method of insertion has been described previously (Harris, 1947). The primary circuits and coils used were of two types: (*a*) a heavy iron cored coil in series with a variable resistance across the a.c. (200 V.; 50 cyc./sec.) mains, and (*b*) a smaller primary coil passing 50 cyc./sec. d.c. pulses produced by the thyratron discharge of a large condenser bank (see Harris (1948*a*)). All stimuli were applied for periods of 1 min., the intensity of the stimulus being gauged by the proximity of the primary coil to the animal's head (bearing the implanted secondary coil), and by the movements observed in the orbital structures due to the spread of current to the adjacent oculomotor nerve. Observations on gastro-intestinal motility were made in the unanaesthetized animals by direct scrutiny of the intact abdominal wall. Rabbits, starved for 24 hr., were tied in the supine position and the fur over the abdomen clipped short, rather than shaved, because the cropped fur made any slight peristaltic waves more obvious. After careful observation of the abdominal wall for at least 10 min., stimulation was performed, or an intravenous injection of 'pituitrin' was given, and observation continued for another 10 or 15 min. The peristalsis normally visible through the abdominal wall of the intact rabbit is somewhat variable. In eleven normal rabbits, observed on many occasions, six showed no peristalsis; three showed occasional, very slight local undulations of the anterior abdominal wall; and only two showed rhythmical waves which started a few minutes after the animal was tied on the table.

Results

Preliminary observations. In the course of some other work in which rabbits were tied supine, it was noticed that stimulation of the neurohypophysis or intravenous injection of 'pituitrin' caused an increase in the peristaltic waves as seen through the abdominal wall. These rabbits had all been subjected to the operation of vaginal transplantation, most of them to ovariectomy, and the subcutaneous implantation of a stilboestrol tablet. That the visible waves observed were not due merely to an increase in uterine motility was clear from their site, viz. to the right of a line drawn from the xiphisternum to the left iliac fossa. In this region the large gut is situated, the part of the intestine which has been described by many workers as being most sensitive to posterior lobe extracts.

It was difficult to make accurate observations on some of these animals, for any increased peristalsis due to stimulation tended to be masked by the very obvious waves present before the stimulus was applied. The prominent intestinal motility normally present in these cases was thought to be due to the oestrogenization, for later observations showed that this increased motility developed a few days or weeks after the operation of vaginal transplantation, ovariectomy and stilboestrol tablet insertion. Other explanations, such as the loss of weight after the operation rendering the peristaltic waves more obvious through a thinned abdominal wall, or interference with the nerve supply of the large intestine by transection of the vagina, were held to be unlikely.

It was therefore decided to investigate the effects of injecting various doses of posterior lobe extract on the peristalsis of normal rabbits and of stimulating various regions of the pituitary gland and hypothalamus by the remote control method.

The effect of intravenous injection of posterior lobe extract on the visible peristalsis of normal rabbits. Six injections of 500 mU. 'pituitrin', eight injections of 200 mU., six injections of 100 mU., three injections of 50 mU. and six control injections of normal saline were distributed amongst eleven normal rabbits. Only one injection was given on any day, except when control injections of saline were previously administered.

50 mU. of 'pituitrin' produced a definite increase in peristalsis in one out of three cases, 100 mU. in five out of six cases, and 200 mU. and 500 mU. in all cases. The responses to the injections were essentially similar in all the animals, and may be described as consisting of three phases:

Phase 1. A latent period of about half a minute.

Phase 2. A stage of increasing and then almost continuous intestinal movement for about two minutes. In most animals, a continuous rolling motion occurred in all parts of the abdomen, so that the general appearance could be likened to a 'bag of worms'. Sometimes short pauses were seen, in which the abdominal wall was still. These gave the impression that a series of waves, each of long duration, was in progress. Once the abdominal wall was observed to be raised into ridges during the pauses, perhaps indicating a state of spasm of the intestines.

Phase 3. A stage of decreasing rhythmical activity. In this phase, rolling motions of the abdominal wall were seen with a frequency of approximately one wave 30–120 sec. and a duration of 10–30 sec.

The effect of stimulation of the hypothalamohypophysial region on the visible peristalsis. Ten rabbits, intact except for the implanted coils, were used in these experiments. On each animal the effect of stimulation was studied on at least three separate occasions. The response of these animals to intravenous injection of posterior lobe extract was also investigated, so that the results of the two methods of stimulation could be compared on the same animal. The site of the stimulating tip of the electrode in these animals could be estimated with fair accuracy during life from a study of X-ray photographs of the head, and by the inhibition of a water diuresis produced by stimulation. It was established exactly, post-mortem, by means of serial sections.

These animals may be classified into three groups according to the position of the electrode: Group A, electrode more than 0·5 mm. from the supraopticohypophysial tract; group B, electrode within 0·5 mm. of, but not in contact with, the supraopticohypophysial tract, and group C, electrode in, or in contact with, the supraopticohypophysial tract.

The results of stimulation are given in Table 1. All the animals (except no. 40) were tested to see the effect of stimulation on a water diuresis, group A animals showing no effect, group B a submaximal antidiuretic response, and group C a maximal response. With regard to the effect of stimulation on peristalsis, similar results were obtained from the animals in both groups A and B. A negative result was observed in thirteen out of eighteen experiments, a doubtful positive response in four, and a marked response in one. The four doubtful responses consisted of a few, small and shortlived, localized undulations that appeared in the 10 min. after stimulation. These waves showed little similarity to those produced by the injection of 'pituitrin' into the same animals. It seems therefore unlikely that they were produced by the spread of current to the neurohypophysis, especially since in rabbits 30 and 32, any spread was insufficient to produce inhibition of a water diuresis which is a far more sensitive reaction. The significance of these four doubtful responses, and of the one definite reaction is unknown. In the group C animals, a definite excitation was observed in thirty-one out of thirty-two experiments, the character of the response being similar in nature to that evoked by intravenous injection of posterior lobe extract. The increased peristalsis occurred after a long latent period (0·5 min. or more after starting 1 min. stimulation), and then appeared as large, almost continuous, undulations mainly situated

Table 1. The effect on peristalsis of stimulating various areas in the hypothalamohypophysial region

Rabbit	Electrode site	No. of experiments	Effect of stimulation on peristalsis 0	±	+	+ +
Group A						
30	Posterior part of tuber cinereum	3	1	2	.	.
32	Right posterior part of tuber cinereum	3	2	.	.	1
40	Zona tuberalis	3	3	.	.	.
Group B						
31	Lateral part of tuber cinereum and pars distalis on right of infundibular stem	3	2	1	.	.
33	Pars distalis below infundibular stem	3	3	.	.	.
34	Posterior part of tuber cinereum	3	2	1	.	.
Group C						
35	In contact with right side of infundibular stem	5	.	.	.	5
45	Median eminence slightly to left	4	.	.	2	2
51	Right side of median eminence	13	1	.	6	6
52	Junction of infundibular stem and infundibular process	10	.	.	2	8

The effect of stimulation on peristalsis is represented thus: 0, no effect; ±, a doubtful increase; +, a definite slight or moderate increase; and + +, a marked increase. All the rabbits responded to the intravenous injection of 100 mU. posterior lobe extract with an increase in peristalsis.

over the colonic area of the abdomen. These waves slowly decreased in frequency and amplitude during the next 5–10 min. The latent period of the response to stimulation was slightly longer than that to injection of 100 mU. of 'pituitrin' into the marginal vein of the ear. Also, as far as could be judged by observations on individual rabbits from day to day, the magnitude of the response to the standard 1 min. stimulation was less than that evoked by the intravenous injection of 100 mU. 'pituitrin' in the same animal. This latter point was observed most clearly in rabbit 52, in which visible peristalsis before stimulation or injection was very slight or absent.

In one rabbit, not included in the above series, after the abdomen had been opened under ether for another reason, the intestine and uterus were observed directly, whilst electrical stimulation of the infundibular stem was performed by the usual method. After stimulation for 45 sec. large peristaltic waves appeared suddenly in the large gut and uterus, both of which had been previously flaccid and quiescent. Though the stimulus ceased after 1 min. the waves continued for at least 4 min., but with decreasing amplitude and frequency. In another animal, observed under similar conditions, peristalsis was not excited by stimulation, possibly because this animal had been longer, or more deeply, anaesthetized.

THE NEUROHYPOPHYSIS AND BLOOD PRESSURE

As regards the production of a pressor response to stimulation of the neurohypophysis in situ, a little evidence is available for animals in the anaesthetized state. Cyon (1898, 1900, 1901) observed a bradycardia and an increase in blood pressure after injection of pituitary extract, or stimulation of the pituitary gland either electrically or mechanically. He suggested that these results were mediated through the vagi. Chang, Chia, Hsu & Lim (1937) reported that stimulation of the central end of the cut vagus in dogs under chloralose anaesthesia, and with only vascular connexions between the head and trunk, gave a pressor response in the trunk. This response could be abolished either by interrupting the pituitary stalk or by removing the pituitary gland. The conclusion drawn was that stimulation of the central vagus induces a reflex secretion of the posterior pituitary principle. Rather more direct evidence is that of Clark & Wang (1939), who found that stimulation of the supraoptic region of the anterior hypothalamus gave a pressor response in seven out of ten anaesthetized spinal cats, the characters of the response being a long latent period ($\frac{1}{4}$–$\frac{1}{2}$ min.), a slow smooth rise of about 30 mm. Hg and a duration of approximately 2–4 min. They therefore thought it likely that the reaction was due to liberation of the posterior pituitary hormone. Sattler (1940) has confirmed, in the main, both the work of Chang *et al.* (1937), and Clark & Wang (1939). Ferguson (1941) records slight rises in blood pressure on stimulating the pituitary stalk in rabbits and cats anaesthetized with chloralose and urethane, and in which only vascular connexions remained between the head and trunk. In some cases, the pressor response was very slight (see his Fig. 2), although the oxytocic effect of the stimulus was well marked.

In view of the fact that the pressor response to injection of posterior lobe extract varies in the conscious and anaesthetized states, it was decided to investigate the effect on the blood pressure of stimulation of the neurohypophysis in the conscious rabbit.

Methods

Systolic pressures were measured on the vessels of the rabbits ears using the method of Grant & Rothschild (1934), care being taken that the vessels were thoroughly dilated by previous warming. With this precautionary measure rabbits gave readings which fluctuated by only a few mm. Hg over long periods. The full technique was as follows. A rabbit, with the outer surface of one ear shaved and the skin over the marginal vein of the opposite ear nicked with a razor, was placed in its cage over an electric heater for usually 4 hr., the temperature in the cage being 81° F. At the end of this period, the animal, with ear vessels well dilated and respiration occurring in shallow rapid pants, was removed from the cage, secured in the upright position in a duster to render it motionless, replaced on the heater and blood-pressure readings taken at about 5 min. intervals till a constant level was obtained. The intravenous injection of posterior lobe extract was then made into the marginal vein of the opposite ear through the previously nicked skin, or electrical stimulation applied for 1 min., and the blood pressure recorded after ½, 1½, 2½, 3½, 5 and 7 min. Stimulation was applied by the same method as used in the previous experiments on peristalsis (see above).

Results

The effect of intravenous injection of posterior lobe extract on the blood pressure of normal rabbits. It was necessary, before investigating the effect of stimulation of the neurohypophysis, to see if the conscious rabbit reacted to an intravenous injection of posterior lobe extract with a pressor response. Fifteen normal rabbits were chosen and, out of these, six different animals were tested at each dosage of 'pituitrin' (50, 100, 200 and 500 mU.), and also with injections of 1 c.c. 0·9 % NaCl solution. In some of the earlier readings the rabbits had been previously given small quieting doses of paraldehyde by stomach tube, but since these results did not differ from those obtained in the later experiments when no sedative was used, all the results have been grouped together. All the doses of 'pituitrin' produced a sharp rise in blood pressure lasting for 30–90 sec., and the pressure then fell more slowly over the next few minutes, the increase in pressure and its duration varying approximately as the dosage (see Table 2).

TABLE 2. The maximum increments in systolic blood pressure after intravenous injection of varying doses of 'pituitrin'

Dose of 'pituitrin' in mU.	500	200	100	50	Control injections
Rise in systolic blood pressure, in mm. Hg	30	28	12	10	5
	32	35	10	5	0
	35	40	10	17	0
	35	20	14	10	0
	33	25	25	10	0
	32	30	17	10	0
Average rise in systolic blood pressure, in mm. Hg	32·8	29·7	14·7	10·3	0·8

A marked bradycardia was also observed after injection of posterior pituitary extract. The figures given by Brown, McLean & Maegraith (1939), who measured the blood-pressure changes of rabbits, anaesthetized with nembutal, after injections of 'infundin' in doses of 0·12 and 0·06 U., varied from 18 to 43 mm. Hg, which are slightly higher than those obtained by the author in the conscious rabbit at an equivalent dosage. Brown *et al.*, however, used the carotid loop method of recording the blood pressure, which might well account for the higher figures, quite apart from their use of anaesthetized animals.

TABLE 3. The maximum rises in systolic blood pressure after stimulation of various areas in the hypothalamohypophysial region for 1 min., and after the intravenous injection of 100 mU. 'pituitrin' in the same animals

		After stimulation		After injection	
Rabbit	Electrode site	Number of observations	Average change in systolic b.p. in mm. Hg	Number of observations	Average rise in systolic b.p. in mm. Hg
Group A					
30	Posterior part of tuber cinereum	3	−2·7	3	15
32	Right posterior part of tuber cinereum	3	−1·0	1	18
40	Zona tuberalis	3	+2·7	2	20
Group B					
31	Lateral part of tuber cinereum, and in pars distalis on right of infundibular stem	3	+2·3	1	25
33	Pars distalis below infundibular stem	3	−2·3	3	15·7
34	Posterior part of tuber cinereum	4	+8·3	3	18
49	Zona tuberalis	2	+2·5	2	6·5
Group C					
35	In contact with right side of infundibular stem	3	+8·3	1	14
45	Median eminence slightly to left	5	+6·8	5	16·6
51	Right side of median eminence	4	+4·3	2	15·5
52	Junction of infundibular stem and infundibular process	5	+8·2	4	16·8

The effect of stimulation of the hypothalamohypophysial region on the blood pressure. The effect of stimulation on the blood pressure was investigated in eleven rabbits bearing implanted coils. Three rabbits fall in group A with the electrode more than 0·5 mm. from the supraopticohypophysial tract, four in group B with the electrode within 0·5 mm. of, but not in contact with, the supraopticohypophysial tract, and four in group C with the electrode in contact with some part of the supraopticohypophysial tract. The results of these experiments are given in Table 3. It may be seen that the average variation in blood pressure after stimulation was very slight in all the animals classified as groups A and B except one (rabbit 34, group B). The four animals in group C seemed to show slight pressor responses (of the same type and time relations as those following injection of posterior pituitary extract), but it is doubtful whether these figures represent a significant physiological response to stimulation of the neurohypophysis. The results show clearly, however, that 100 mU. of whole posterior lobe extract injected intravenously produced greater pressor responses than stimulation of the neurohypophysis for 1 min.

OXYTOCIC RESPONSES ELICITED BY STIMULATION

Some of the rabbits used in the experiments described above were investigated to ascertain the effect of similar stimuli in eliciting the secretion of an oxytocic principle. Stimulation was applied for 1 min. in the usual way. The uterine responses of the unanaesthetized rabbits were recorded, from a permanent vaginal fistula, in animals that had been ovariectomized and implanted subcutaneously with a 50 mg. tablet of stilboestrol di-*n*-butyrate (see Harris, 1947, for a full description of the technique).

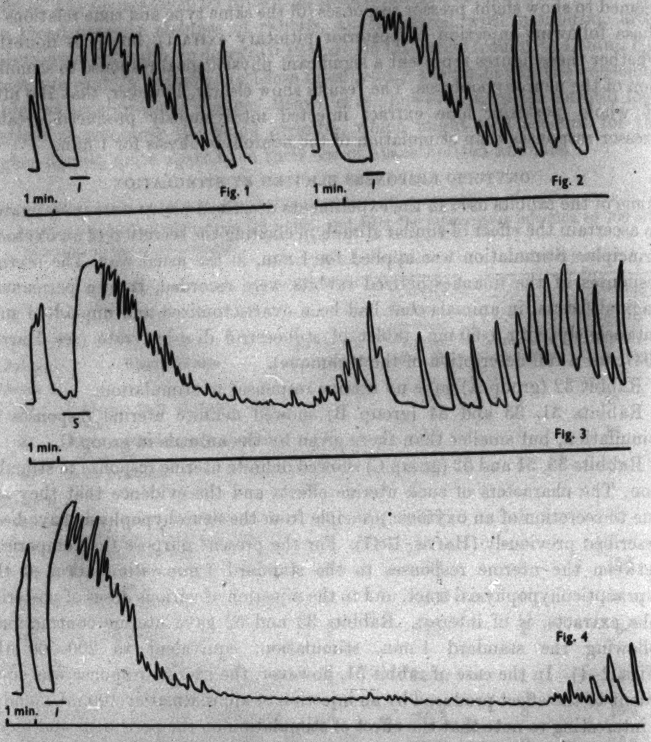
Rabbit 32 (group A) gave no uterine responses to stimulation.

Rabbits 31, 33 and 34 (group B) showed definite uterine responses to stimulation, but smaller than those given by the animals in group C.

Rabbits 35, 51 and 52 (group C) showed definite uterine response to stimulation. The characters of such uterine effects and the evidence that they are due to secretion of an oxytocic principle from the neurohypophysis have been described previously (Harris, 1947). For the present purpose the comparison between the uterine responses to the standard 1 min. stimulation of the supraopticohypophysial tract, and to the injection of various doses of posterior lobe extracts, is of interest. Rabbits 35 and 52 gave uterine contractions, following the standard 1 min. stimulation, equivalent to 200–500 mU. (Figs. 1–4). In the case of rabbit 51, however, the uterine response was equivalent to the effect produced by an injection of approximately 100 mU., and it is interesting to note that the effect of stimulation on the peristalsis and blood pressure in this rabbit had also appeared to be less than that obtained in rabbit 52. The probable reason is that in rabbit 51 the electrode was situated in contact with the right side of the supraopticohypophysial tract in the median eminence where the tract is splayed out as a flat band of fibres, whereas in rabbit 52 the electrode was in the middle of the fibres where they are concentrated as a round bundle in the junction of the infundibular stem and infundibular process. It is likely then that only a proportion of the supraopticohypophysial fibres were stimulated in rabbit 51.

DISCUSSION

The results show that the conscious rabbit reacts to the intravenous injection of small doses of whole posterior pituitary extract with, amongst other effects, an increase in intestinal peristalsis and a pressor response. Stimulation of the



Figs. 1–4. Oxytocic responses of rabbit 52 (group C) to intravenous injection of posterior lobe extract, and to stimulation of the infundibular stem. In comparing these curves attention should be paid to the initial phase of contraction of the uterus, and in particular to the general shape of the curve, and the tendency to maintain a plateau at the height of the contraction. The phase of inhibition that follows the initial contraction, after stimulation of the neurohypophysis or injection of whole posterior lobe extracts, is discussed in the text.

Fig. 1. Intravenous injection 100 mU. 'pitocin', *I*.

Fig. 2. Intravenous injection 250 mU. 'pitocin', *I*.

Fig. 3. Stimulation of neurohypophysis, *S*.

Fig. 4. Intravenous injection 250 mU. 'pituitrin', *I*. (This curve is not quantitatively comparable with the other curves.)

(Tracings of kymograph recordings × ½).

neurohypophysis causes a similar increase in peristalsis, but no marked rise in blood pressure. Before assigning a physiological rọle to the neurohypophysis in the control of intestinal peristalsis it would be necessary to demonstrate that lesions of the neurohypophysis interfere with the normal motility of the intestine, or prevent an increase in motility normally evoked by 'natural' stimuli. In this respect Cushing's (1932) observation that '...patients with tumors which have destroyed the pars nervosa or thrown it out of function by compression are, in many instances, notably victimized by chronic constipation', is of interest.

Evidence is accumulating that stimulation of the neurohypophysis in the conscious rabbit excites a secretion containing less pressor than oxytocic principle, as compared with unfractionated posterior lobe extracts.

(1) It has been shown (Harris, 1947) that when the oxytocic and antidiuretic responses are measured simultaneously, the oxytocic response elicited by stimulation of the neurohypophysis is greater relative to the antidiuretic than that evoked by injection of whole posterior lobe extracts. It is known that the antidiuretic activity of such extracts parallels the pressor activity.

(2) Stimulation of the neurohypophysis has been shown to produce an oxytocic effect equivalent to that produced by 200–500 mU. of whole posterior lobe extract, whereas similar stimulation in the same animals produces an effect on the intestinal peristalsis and the blood pressure less than that evoked by 100 mU. of whole posterior lobe extract. (It is likely that, in the rabbit at least, the peristalsis-stimulating action of whole posterior pituitary extracts is due to the pressor principle.) Further evidence on the amount of pressor substance liberated from the gland following stimulation for 1 min. is given by the fact that less antidiuretic hormone is excreted in the urine after such a stimulus than after the intravenous injection of 100 mU. 'pituitrin' (Harris, 1948*b*).

(3) Stimulation of the neurohypophysis excites an oxytocic response which is more closely simulated by intravenous injection of 'pitocin' than 'pituitrin' (Ferguson, 1941; Harris, 1947), the 'pituitrin' response showing a greater inhibition of the rhythmic waves after the initial contraction (see Figs. 1–4). The inhibitory phase following the response to stimulation is found to be intermediate in magnitude between that following injection of 'pitocin' and injection of 'pituitrin'. This inhibition is due to the pressor principle (Morgan, 1937; Harris, unpublished), probably causing spasm of the uterine arteries (Morgan, 1937). Thus the evidence derived from the uterine tracings alone strongly suggests that the pituitary secretion, excited by electrical stimulation, contains relatively less pressor principle than whole posterior pituitary extracts.

The evidence given above should not be used to support the 'unitary theory' or 'multiple hormone theory'. Two questions are involved here. First, does the gland manufacture a single substance with multiple actions, and secondly, if a single substance with multiple activities is formed in the gland, is it liberated as such into the blood stream, or as suggested by Van Dyke, Chow, Greep & Rothen (1942), are active fragments of the parent molecule discharged into the blood stream separately according to the requirements of the organism? It should be mentioned that Van Dyke *et al.* (1942) succeeded in isolating a protein from the dried posterior lobe of frozen gland material which behaved as a homogeneous substance and had oxytocic, vasopressor and diuresis-inhibiting activities present in ratios resembling those of the U.S.P. reference standard.

It was stated (Harris, 1944, 1947) that if it could be shown that the proportions of pressor (and/or antidiuretic) and oxytocic activities as produced by stimulation of the neurohypophysis ran parallel under a variety of conditions (hydration and dehydration, anoestrus, oestrus, pregnancy, pseudopregnancy and post-partum, etc.), then strong evidence would be obtained for the 'unitary theory'. However, preliminary experiments indicate that such is not the case. It has been found that the reactions of the oestrogenized uterus to intravenous injection of a solution containing 'pitocin' and 'pitressin' may give a roughly quantitative assay of the 'pitocin' and 'pitressin' content of such mixtures. The 'pitocin' content is related to the initial uterine contraction, and the 'pitressin' content to the subsequent inhibitory phase. Stimulation of the infundibular stem for 1 min. produces a secretion equivalent (in action on the uterus) to a mixture of 250 mU. 'pitocin' and 50–100 mU. 'pitressin'. Now since administration of hypertonic saline has been shown to excite the liberation of the antidiuretic hormone from the posterior pituitary gland (Chambers, 1945; Verney, 1946), the effect of stimulation one hour after intravenous administration of hypertonic saline was investigated. Under these conditions the stimulus appears to produce a secretion equivalent in action to the same amount of 'pitocin', but less 'pitressin'. Since the hypertonic saline does not alter the uterine reactions to intravenous injection of posterior pituitary extracts, the simplest explanation seems to be that the injection of hypertonic saline has depleted the neurohypophysis of its pressor content, but not the oxytocic. Further work is in progress on this subject.

The effect of whole posterior pituitary extracts, or of the pressor principle, in causing a phase of inhibition of uterine motility following any initial contraction appears to be due to the production of a state of spasm of the uterine vessels (Morgan, 1937). If this explanation is the true one, the fact that stimulation of the neurohypophysis is followed by a similar, though smaller, reaction demonstrates that the secretion of the gland can produce an effect on, at least, the uterine blood vessels. The question then arises as to why an effect on the general vascular tree was not demonstrable as a definite pressor response. Of many possible explanations the most likely appear to be (*a*) that the uterine vessels are specifically sensitive to the action of the pituitary secretion, (*b*) that nervous reflexes mask a pressor response otherwise due to a general vasoconstriction (though a bradycardia has not been observed to follow stimulation of the neurohypophysis), and (*c*) that the uterine responses were recorded in oestrogenized rabbits, whereas the pressor effects were investigated in non-oestrogenized animals. There can be no doubt that oestrogens sensitize the uterine musculature of the rabbit to the action of whole posterior pituitary extract and to the secretion liberated by stimulation of the neurohypophysis. They seem to cause also an increase in the intestinal peristalsis of the rabbit as seen through the intact abdominal wall, which would appear to be an effect exerted on the plain muscle of the intestine. With regard to a possible sensitization of vascular muscle, Byrom (1937, 1938) found an increased sensitivity of the blood vessels of rats to the pressor principle by the previous administration of oestrogens. This increase in sensitivity was of the order of a tenfold, or greater, increase. A few observations were made on the effect of stimulating the neurohypophysis of rabbit 35, after the operation of vaginal transplantation, ovariectomy and subcutaneous insertion of a 50 mg. tablet of stilboestrol di-*n*-butyrate. In three experiments the systolic blood pressure showed a rise of 32, 20 and 24 mm. Hg (average rise 25·3 mm. Hg) as compared with rises of 15, 7 and 3 mm. Hg (average rise 8·3 mm. Hg) before the operation. Further evidence on this point is needed.

SUMMARY

1. Intravenous injection of posterior pituitary extract (50–500 mU.) causes an increase in the peristalsis visible through the abdominal wall, and electrical stimulation of the neurohypophysis causes an increase similar in type, though less in magnitude, to that produced by 100 mU. of such extracts in the same animals.

2. Intravenous injection of posterior pituitary extract (50–500 mU.) causes an increase in systolic blood pressure (10–30 mm. Hg), and stimulation of the neurohypophysis causes a slight rise of systolic blood pressure of doubtful significance, and clearly smaller, than that produced by 100 mU. of such extracts in the same animals.

3. Stimulation of the neurohypophysis (in the same and other animals as those used in the above experiments) produces an effect on the uterus equivalent to 200–500 mU. of posterior pituitary extract.

4. The nature of the substance liberated from the neurohypophysis by electrical stimulation is discussed.

It gives me pleasure to acknowledge the kindness and encouragement of Prof. H. A. Harris. My thanks are also due to Mr L. C. Maltby for making the implanted units, and to Mr R. Smith for his assistance and his care of the animals. The expenses of this research were in part defrayed by a Parliamentary Grant-in-Aid administered by the Royal Society.

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THE STANDARDIZATION OF SECRETIN AND PANCREOZYMIN

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This work was the outcome of attempts to determine activity in some preparations of secretin. When the first preparation was injected intravenously into a cat under chloralose it failed to produce a secretion of pancreatic juice, and to ensure that the pancreas was capable of responding, a freshly prepared extract from the duodenal mucosa of a dog was used as a control. For further experiments an acetone-dried powder was made by the simple procedure originally suggested by Smith & McClosky (1924) for keeping posterior pituitary material in a stable form.

METHOD

The small intestine, including the pylorus, was excised, washed quickly under the cold tap to remove blood, and the first 5 ft. was taken (see Table 1). This was slit longitudinally, and the mucosa washed with cold water. The mucosa was scraped off with a blunt scalpel, minced in a machine and weighed on paper. It was transferred to a beaker and put in the freezing chamber. The procedure thus far was complete within 10 min. When frozen solid, the mince was ground in a mortar surrounded by a freezing mixture of ice and salt. Ice-cold acetone was poured on to this, mixed and the mixture filtered on a Buchner funnel using a filter pump. This extraction was performed three times in succession, after which the mucosa appeared finely divided. The material was spread in a dish and left in an evacuated desiccator over $CaCl_2$. After 24 hr. the dry material was ground in a mortar and passed through a no. 100 sieve. It was stored in a dark bottle.

To obtain a preparation likely to be stable for a long time the material was extracted with acetone in a Soxhlet apparatus for 4 hr., and kept in vacuo over P_2O_5 for 24 hr. It was then distributed into small tubes which were left in vacuo over P_2O_5 for 2 days. The desiccator was filled with dry air and the tubes were taken out and sealed immediately. They were kept in the dark at 4° C.

A large amount of powder was obtained in this way and from it an extract was prepared before each experiment as a standard with which to compare unknown samples. The extract was prepared as follows: 1 g. powder was mixed with a small portion of 25 ml. 0·15 N-HCl in an evaporating dish to make a smooth paste. The rest of the 25 ml. was added. The dish was put over a low flame and the contents brought to boiling-point in about 5 min. The mixture was stirred and allowed to boil for exactly 60 sec. The flame was then removed, red litmus paper added and N-NaOH (usually 3·5 ml.) run in with stirring until coagulation of the protein mass occurred and the litmus paper turned purple. The time taken for neutralization was 30 sec. The extract was filtered through a folded paper into a 25 ml. measuring cylinder. The protein mass and filter-paper were washed with a few ml. of distilled water and the washings were filtered into the measuring cylinder. The volume of filtrate was made up to 25 ml. 1 ml. thus corresponded to 40 mg. acetone-dried powder. This extract was found to lose activity after 24 hr.

Method of comparison. Several samples of acetone-dried powder were prepared, and were compared with a sample of secretin very kindly provided by Dr C. L. G. Pratt, which will hereafter be referred to as sample A. The comparisons were made in cats weighing 1·8–3·5 kg. lightly anaesthetized with chloralose. Unless the first sample of pancreatic juice was required, it was found advisable to give an injection of secretin immediately before cannulating the pancreatic duct, to wash out traces of blood and to enable the best position to be chosen for the cannula. The abdomen was closed after clamping the cannula. In some experiments the drops were recorded by the Gaddum drop-timer (Gaddum & Kwiatkowski, 1938), the pancreatic juice displacing tap water from a reservoir.

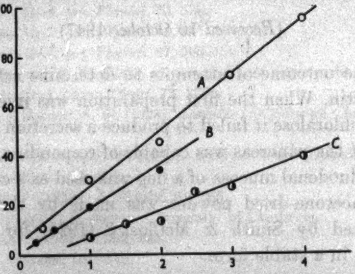

Fig. 1. Relation between dose and volume of juice in three experiments. Line *A* was obtained by using sample A, lines *B* and *C* by using powder D4. Ordinates: drops of pancreatic juice. For line *A* the abscissae are mg. sample *A*. For lines *B* and *C* the abscissae are ml. extract, 1 ml. containing the activity of 40 mg. powder D4.

RESULTS

The relation between the dose injected and the number of drops of pancreatic juice secreted was found to be linear as shown for three experiments in Fig. 1. Line *A* was obtained by the injection of sample A; lines *B* and *C* were obtained with extracts of the same acetone-dried powder D4 in different cats. The greater effect of sample A in Fig. 1 depended on the ready response of the particular cat used, and no importance attaches to the relative position of the lines.

Variation in different powders. Powders were prepared from seven dogs and two cats. These were compared with sample A used as a standard, each comparison being made side by side in the same cat. Usually the dose-response curve for the standard was constructed, and, when the effects of two different doses of the test extract were observed, the amount of extract equivalent to a given weight of standard was determined from the curve. The potencies of different powders, given in Table 1, were found to depend on the portion of intestine used (as has been repeatedly observed), but it was surprising that preparations as active as D7*c* were obtained by so simple a process. The extracts produced a transitory fall of blood pressure when injected; but even a large dose, equivalent to 120 mg. powder, was not more depressant than 0·02 mg. histamine. The fall was almost certainly due to histamine, as it was unaffected by atropine, and in a cat in which the fall was followed by a rise, the rise disappeared when the suprarenals were excluded. This is characteristic of histamine (Burn & Dale, 1926).

TABLE 1. **Relative activity as secretin of different acetone-dried powders of duodenal mucosa**

Powder	Part of intestine used	Amount ≡ 1 mg. Sample A (mg.)
Dog D1	All small intestine	>300
„ D2	All small intestine	300
„ D3	First 5 ft.	40
„ D4	First 5 ft.	40
„ D5	First 1·25 ft.	16
„ D6	First 4 in.	30
„ D7*a*	First 4 in.	14
„ D7*b*	Second 4 in.	14
„ D7*c*	Third 4 in.	10
„ D7*d*	Fourth 4 in.	25
Cat C1	All small intestine	200
„ C2	All small intestine from 3 cats	100

Pancreozymin in acetone-dried powders. The powders were next examined for the presence of pancreozymin, the hormone described by Harper & Raper (1943) which stimulates the output of enzymes. This was done by testing the pancreatic juice for amylase by Wohlgemuth's method (Cole, 1941). When sample A was injected the first juice obtained was relatively rich in amylase, but the concentration of amylase fell rapidly in response to further injections. When this concentration had reached a negligible level, an injection of acetone powder extract, which produced the same rate of flow of juice, caused the concentration of amylase to rise.

This effect is illustrated in Fig. 2, in which the logarithm of the diastatic power has been plotted as ordinate against the volume of juice secreted. (The diastatic power was determined as the volume of 1% starch converted to erythrodextrin by 1 ml. of the enzyme solution in 30 min. at 40° C.) In this experiment there was an initial spontaneous secretion which was unusual; the amylase content of this juice was high. After 2 ml. had been secreted, the juice was obtained only in response to injections of secretin. The injection of sample A began at point *A* in Fig. 2 and produced juice with a low concentration of amylase. Eight injections of sample A were given during 80 min., and the juice remained low in amylase. The test extract was then injected and the concentration of amylase at once rose; a rise was also obtained after each of two further injections, the third being given after 2 mg. atropine sulphate.

Five experiments of this kind were done, a rise in amylase concentration occurring after each injection of extract of acetone-dried powder in every experiment.

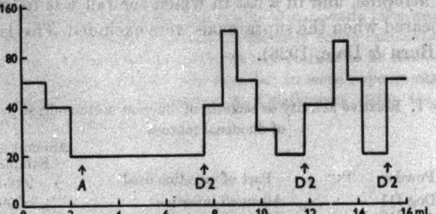

Fig. 2. The increase in concentration of amylase in the pancreatic juice after injections of extract of acetone-dried powder. Ordinates: log diastatic power. Abscissae: volume of juice, in ml., secreted since the beginning of the experiment. Before point *A* the secretion was spontaneous. At point *A* the first injection of sample A was given and the flow of juice was maintained by regular injections of sample A except where the test extract was injected at D2. The third injection of D2 was given after 2 mg. atropine.

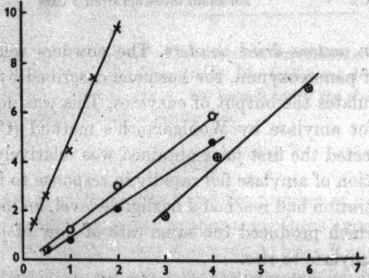

Fig. 3. The relation between dose of pancreozymin and amount of amylase secreted in four experiments. Ordinates: volume × log diastatic power. Abscissae: ml. extract of D3 injected. (1 ml. corresponds to 40 mg. powder.)

Relation of dose to amylase secreted. Experiments were then performed to discover whether the amount of amylase was proportional to the dose of extract. In these sample A was injected until the diastatic power became 20 or less, and then different doses of extract were given. As these doses increased, the volume of juice increased; in some experiments the concentration of amylase rose, in others it remained about the same and in one it fell. In all, however, the amount of amylase, determined as the product of the concentration and the volume, rose as the dose increased, and there was a linear relation between the product 'log diastatic power × volume' and the dose of extract. The relation is shown for four experiments in Fig. 3, and provides a basis for comparing samples of pancreozymin.

DISCUSSION

An acetone-dried powder was first proposed as an international standard preparation for pituitary (posterior lobe) extract as a result of the work of Smith & McClosky (1924). Acetone-dried powders have since been prepared from anterior lobe and recently from brain and from liver. The evidence described in this paper shows that secretin and pancreozymin are preserved in a powder so prepared from duodenal mucous membrane. In 1912, Stepp remarked, at the end of a summary to his paper: 'No secretin is extracted by acetone, ether or absolute alcohol. This fact may be utilized to prepare a dried powdered mucous membrane which may be kept permanently, and will serve for the preparation of secretin at any time by boiling with dilute acid.' He did not, however, describe experiments carried out with such powders.

Secretin, which was discovered nearly 50 years ago by Bayliss & Starling (1902), is now sold commercially, but there is still no international standard for this substance though it was the first to be called a hormone. Various units have been proposed, and some of these are obviously unsatisfactory to those familiar with the development of biological standardization during the last 25 years. Seager (1939) defined a unit of secretin as 'the amount of gall bladder contracting material present in 0·2 c.c. of solution which, when injected intracardially into 30 g. frogs, brings about contraction in 50% of 30 experiments'. Ivy, Kloster, Drewyer & Lueth (1930) defined a 'threshold dose of secretin' and 'that amount of dried material in solution which when injected intravenously will cause a 10-drop (0·4 c.c.) increase in the rate of flow of pancreatic juice within a 10 min. period following the time of injection as compared with the preceding 10 min. period'.

Ivy *et al.* (1930) state that dogs vary widely in their response to secretin, but that the threshold response is less variable than the response to higher doses. The explanation of this is given in Fig. 1, which shows the response of different cats to injected secretin. It can be seen that the amount of juice produced in response to any one dose varied from cat to cat, and that the variation in threshold was less but, nevertheless, existed. For instance, the doses of secretin producing a response of 10 drops (a threshold response) in the three experiments shown were equivalent to 0·25, 0·6 and 1·3 mg. sample A.

Greengard & Ivy (1945) have arrived at a unit of pancreozymin in the following way. 'A dog with the pancreatic duct cannulated is given repeated injections of secretin in a dosage adequate to elicit about 3 c.c. of pancreatic juice in 15 min., and the collections from the first four injections are discarded. The responses to the 5th and 6th injections are divided into 3 min. fractions and kept for enzyme analysis, and on the 7th and 8th injections the pancreozymin is added to the secretin, the juice collected is similarly fractionated and the enzyme content compared with that of the control samples.' 'The unit of pancreozymin may be defined as the amount which will raise the enzyme content of the second 3 min. fraction to the level of the first.' Experience with all the hormones with which much quantitative work has been done has shown that units defined in this way have always proved unsatisfactory. The biological response to a given amount of any hormone has always been found to vary from one animal to another.

The estimation of an unknown sample of secretin or pancreozymin should be made by comparing it with a standard preparation in the same animal. This procedure has been followed for secretin by Still (1931), who used a 'potent vasodilatin-free preparation which has been preserved as our standard, and to which all preparations are compared'. Still injected the standard, and followed this by a dose of test which produced a similar response, and then repeated the standard. He took the ratio of activity to be the same as the ratio of drops produced. He appears to have assumed that the slope of the line relating dose to response was the same for each animal; our results show that the slope varies and should be determined in each experiment. It is not clear from Still's paper how he defined his unit. If a standard is available, the unit should be defined as the activity present in some arbitrarily selected weight of it. Should experiment indicate that 1 g. of an unknown is three times as strong as 1 g. of the standard, then if the unit is the activity in, for example, 0·1 g. of the standard, the unknown contains 30 units/g.

The need for a standard preparation and an international unit is evident from the frequent references to 'clinical units' of secretin in the literature. An acetone-dried powder might be used as a standard preparation for both secretin and pancreozymin. Care must be taken to carry out the drying thoroughly if the preparation is to be stable. Powders prepared without the Soxhlet extraction and kept in an ordinary bottle were found to lose about 80% of their secretin activity in 6 months. When, however, full precautions were taken to remove all fat and moisture during the preparation, and to keep the product dry in sealed tubes, the secretin activity was maintained in full for more than 3 months, as shown by a careful comparison with sample A. A powder kept over sulphuric acid in vacuo lost activity in 5 days. An international committee considering this question might prefer to prepare a standard for secretin as pure as possible, and another similar standard for pancreozymin, but a provisional standard for both can be obtained without delay by the simple means described.

One other use remains for acetone-dried duodenal mucosa, namely for students; they might learn to prepare it, and to demonstrate its action as described by Sherrington (1919).

SUMMARY

1. Acetone-dried powder prepared from the duodenal mucous membrane of dogs and of cats has been found to contain both secretin and pancreozymin.

2. When an acid extract is made from this powder it is found that there is (*a*) a linear relation between the dose and the volume of pancreatic juice; (*b*) a linear relation between the dose and the product of the logarithm of the diastatic power and the volume of juice. These observations provide a basis for the quantitative comparison of samples of secretin and pancreozymin.

3. The need for an international standard preparation for secretin and pancreozymin is stressed. An acetone-dried powder could be used.

Our thanks are due to Dr C. L. G. Pratt for a sample of secretin.

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THE SOUNDS FROM SINGLE MOTOR UNITS IN A CONTRACTING MUSCLE

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Most people are familiar with the roaring sound which may often be heard in their ears during strong contraction of the jaw muscles when the external meatus of the ear is blocked at its outer end. We have, however, been able to hear in quiet surroundings a succession of discrete clicks or pops occurring during much slighter degrees of muscular contraction. These clicks occur with a fairly regular rhythm whose frequency varies with the strength of the contraction. We were struck by the resemblance of these rhythms to a loudspeaker reproduction of the action currents from single motor units, and it seemed probable that the sounds were the mechanical counterparts of the muscle action currents. This paper deals with the relation between these two phenomena.

APPARATUS AND METHODS

All the experiments to be described were carried out on intact human subjects.

Whenever muscle action potentials were recorded, they were picked up through the skin by means of bipolar electrodes. The electrodes consisted of a pair of parallel wires of enamelled constantan between 0·06 and 0·12 mm. in diameter. The ends of the wires were cut square, and the flat uninsulated surfaces were applied to the skin, with the axis between their centres in line with the main axis of the muscle fibres. The separation of the electrodes varied, in different experiments, between 0·1 and 1·5 mm. The subject was earthed through a silver plate which he held in his mouth, and this procedure reduced interference from the mains enough to make the use of a shielded room unnecessary.

When investigating the mechanical disturbances underlying the clicking sounds, two types of electro-mechanical instrument were used. The sounds were recorded by means of a crystal microphone. Alternatively, a stiff piezo-electric system, in direct contact with the skin, was used to record mechanical movements of the underlying muscle, without the intervention of a column of air. In order to record simultaneously the electrical action currents and the mechanical effects, a pair of wire electrodes, like those just described, was incorporated in each of these instruments. The potentials from the wire electrodes and from the microphone or mechanical device were led separately to two resistance-capacity coupled push-pull amplifiers having degeneration of in-phase signals. The amplifier used for action potentials has an overall time constant of about 10 msec., and the one used for recording sounds and movements has an overall time constant of about 0·5 sec. When, however, a piezo-electric recording instrument forms part of the input circuit of this second amplifier, the capacity of the crystal greatly reduces the time constant of the whole system (see below).

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The apparatus for the simultaneous recording of action potentials and sounds is shown in Fig. 1. It consists of a pair of wire electrodes (*B*) in the middle of a microphone orifice, which are held in position by a narrow strip of rubber (not shown in the figure). This orifice fits in an airtight fashion on the skin (*A*). The microphone chamber (*C*) is closed at its other end by the diaphragm (*D*), thus forming a completely enclosed space. Movements of the diaphragm deform the Rochelle salt crystal (*E*), which has piezo-electric properties, and therefore gives rise to potentials which are led through screened cable to the amplifier (*F*).

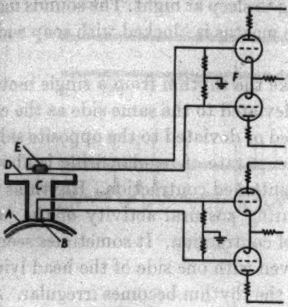

Fig. 1.

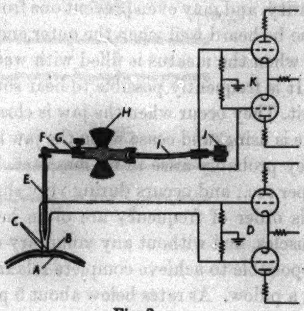

Fig. 2.

Fig. 1. Apparatus for the simultaneous recording of action potentials and sounds (for explanation, see text).

Fig. 2. Apparatus for the simultaneous recording of action potentials and muscle movements (for explanation, see text).

The apparatus for the simultaneous recording of action potentials and movements through the skin is shown in Fig. 2. It records the electrical action potentials arising under the ends of a pair of wire electrodes (*C*), and at the same time records the mechanical force exerted on both the flat ends by the underlying muscle (*A*). This is achieved by connecting the two electrodes through screened cable to one amplifier (*D*): at the same time a rod (*E*), to which the electrodes are rigidly attached, actuates a crystal gramophone pick-up whose output is connected to the other amplifier (*K*). The rod (*E*) is 30 mm. long and 1 mm. in diameter, and is made of a magnesium alloy. The electrodes are attached to the sides of the rod with silk thread and shellac, and the ends project 0·5 mm. beyond the lower end of the rod on to the skin (*B*). The upper end of the rod, which has a flat head like a nail, bears against the spherical knob at the end of a second rod (*F*) which is 20 mm. long, and also of magnesium alloy. In this way a universal joint is formed, and for convenience of manipulation the two members of the joint are held together with a treacly mixture of castor oil and resin. The second of these rods is inserted in the chuck (*G*) of a Rothermel piezo-electric gramophone pick-up ('bender' type). In the pick-up, the chuck member rotates on rubber bearings (*H*), and is attached by means of rubber pads to the free end of the Rochelle salt crystal (*I*). The other end of the crystal is clamped between a second pair of rubber pads (*J*). The time constant of the crystal, screened leads, and the pair of 1 MΩ. grid leaks was found experimentally to be 15 msec.

When this apparatus is used for investigating the facial muscles (e.g. m. orbicularis oculi), the subject lies on his back with his head in a moulded plaster support. A loop, or 'halo', of lead piping is rigidly attached to this support, and the pick-up is fixed to the part of the loop which overhangs the appropriate muscle by means of a rack-and-pinion mounting. This allows fine adjustment of position.

RESULTS

The sounds heard by the ear during the contraction of adjacent muscles

One of the simplest ways of hearing the muscle sounds is to close the external meatus at its outer end, for example, by lying with one's head on a pillow. Once one has learnt to recognize these sounds, they are heard in this way with great clarity, and may even prevent one from going to sleep at night. The sounds may also be heard well when the outer end of the meatus is blocked with soap suds, or when the meatus is filled with water.

It is frequently possible to hear sounds like the rhythm from a single motor unit. They occur when the jaw is closed or deviated to the same side as the ear one is using, and cease when the jaw is opened or deviated to the opposite side: they probably arise in m. masseter. The lowest rate of regular firing is about 5 per sec., and occurs during very slight maintained contraction. Rhythms of this order of frequency are often heard during postural activity of the jaw muscles, and without any voluntary effort of contraction. It sometimes seems impossible to achieve complete relaxation even with one side of the head lying on a pillow. At rates below about 5 per sec. the rhythm becomes irregular. As the strength of contraction increases the rhythm from a single unit becomes faster and may be distinguished up to 10 or 15 per sec., but becomes involved in a rapidly mounting roar as more and more units become active and increase their rates of firing. One of us is able to hear very distinctly the sounds from m. auricularis superior when the pinna is raised in voluntary contraction, but sounds from a single unit can only be distinguished in one ear. This particular unit can be followed even during a maximal contraction, which suggests that it happens to be very favourably placed anatomically for conducting its sounds to the ear. It was possible, by means of a miniature microphone inserted a short distance into the meatus, to make a record (see Fig. 3*a*) of the sounds of a maximal contraction of this unit. The best results were obtained when the canal was partially filled with soap suds. The sounds were, of course, simultaneously heard by the subject, the microphone and soap suds effectively closing the meatus.

It is not always entirely clear how these sounds are conducted to the internal ear. It seems most probable that the motor units cause movements of the walls of the meatus, and that these in turn cause pressure changes in the meatal air-column which actuate the ear drum. The following observations support this theory. The sounds are only heard when muscles in the immediate neighbourhood of the meatus are contracted. Blocking the external end of the meatus makes the sounds audible, presumably because the air-pressure changes, due

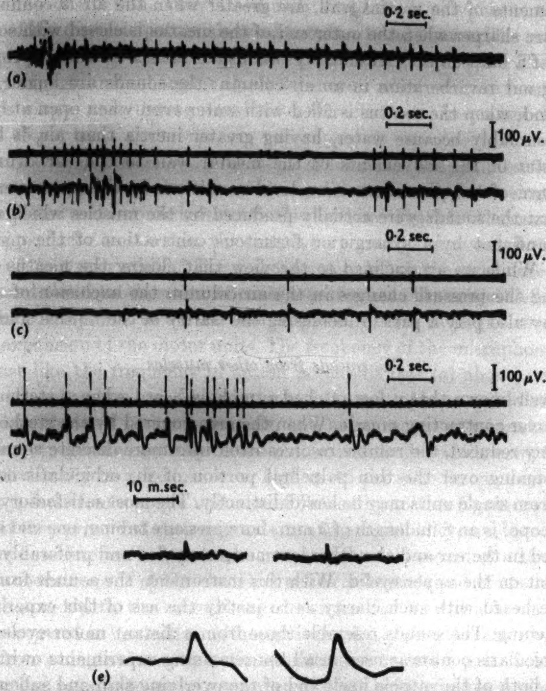

Fig. 3. (*a*) Record of sounds picked up by a microphone inserted in the external meatus of the human ear. A single sustained contraction of m. auricularis superior occurs during the record. The large complex oscillation at the beginning is an artefact caused by the slight displacement of the microphone which occurs as the pinna moves upwards. The sounds from one motor unit stand out clearly throughout the contraction. (*b*) Simultaneous record of action potentials (top line, bipolar electrodes 1 mm. apart) and of sounds (bottom line, microphone with 6 mm. diam. orifice) taken from the upper eyelid (m. orbicularis oculi). Two light closing movements of the eye occur during the record, the first stronger than the second. The activity of the same motor unit stands out in both lines. The scale of μV. refers to the top line. (*c*) As (*b*) above. Four very slight movements were made, as though to close the eye: each movement is represented by one action potential. (*d*) Simultaneous record of action potentials (top line, bipolar electrodes) and of mechanical movements (bottom line, piezo-electric instrument), each taken from the same small area of contact with the upper eyelid (electrode separation 0·2 mm.). The eye was kept closed throughout, the tension being steadily increased, then relaxed, and finally increased again slightly, giving rise to the final action potential. The activity of the same motor unit stands out in both lines. The scale of μV. refers to the top line. (*e*) As (*d*) above. Two records taken at higher speed, to show the relation in time of an action potential (top line) to its accompanying mechanical movement (bottom line).

to movements of the meatal wall, are greater when the air is confined. The sounds are sharper when the outer end of the meatus is closed with soap suds instead of a hard object, presumably because of their well-known property of damping out reverberation in an air-column: the sounds are louder, on the other hand, when the meatus is filled with water even when open at its outer end, presumably because water, having greater inertia than air, is a better transmitter of the movements of the meatal wall to the ear drum. This dependence of the phenomenon on local conditions in the meatus makes it likely that the sounds were actually produced by the muscles which we have named, and not by a synergic or fortuitous contraction of the middle ear muscles. While we are inclined to the view that closing the meatus acts by increasing the pressure changes in the air-column, the exclusion of external noise may also play a part in increasing the clarity of the muscle sounds.

The sounds from other muscles

It is well known that a low-pitched rumble is heard when a stethoscope is applied over contracting muscle. When the area covered by the stethoscope is sufficiently reduced, the rumble resolves itself into more discrete sounds, and, when listening over the thin palpebral portion of m. orbicularis oculi, the sounds from single units may be heard distinctly. The most satisfactory form of 'stethoscope' is an 8 in. length of 3 mm.-bore pressure tubing, one end of which is inserted in the ear and the other, cut accurately flat and preferably wetted, placed flat on the upper eyelid. With this instrument, the sounds from single units are heard with such clarity as to justify the use of this experiment in class teaching. The sounds resemble those from a distant motor-cycle.

M. orbicularis oculi was used in all the remaining experiments owing to the thinness both of the muscle itself and of the overlying skin and subcutaneous tissue. Records of the sounds from single units were never obtained so clearly from any other muscle investigated, even in the face region, and the thicker and deeper-lying muscles of the limbs only gave a complex rumbling sound.

The correlation of the electrical and the mechanical events in a contracting motor unit

(*a*) *The recording of electrical action potentials.* When using the bipolar electrodes previously described, it is easy to obtain records from single units of m. orbicularis oculi. It is unnecessary to clean the skin or prepare it in any other way. After the upper eyelid has been fully exposed by stretching it downwards and fastening with adhesive cellulose tape, the electrodes are pressed firmly against selected areas. The subject then makes graded voluntary contractions of the muscle.

The recorded potentials from single motor units are largest when the electrodes are near the inner end of the eyelid, and may reach 0·2 mV. with an interelectrode gap of 0·2 mm. They are usually diphasic or polyphasic, the total duration of the diphasic potentials lying between 1 and 2 msec. The activity of a particular unit may usually be followed for as long as desired. The lowest frequency of regular discharge is about 3 per sec.: when we have been able to observe a single unit during maximal contraction its frequency has not exceeded 45 per sec.

(*b*) *The recording of the sounds from single motor units.* When the orifice of the microphone is applied to the skin of the eyelid the sounds from single units can be amplified and recorded on a cathode-ray tube. Provided the microphone orifice fits perfectly on the skin, the sounds may usually be recorded well from any part of the upper eyelid.* The spikes as recorded on the screen are usually diphasic and the total duration of each is 5–15 msec. The initial deflexion in each spike corresponds to an increase in pressure such as might result from a lateral expansion of the motor units. The frequency of the microphone spikes varies, just like the frequency of action potentials (see (*a*) above), with the strength of contraction: as the durations of the microphone spikes are much greater than those of action potentials, and as no record in a maximal contraction is ever confined entirely to one unit, it is difficult to follow microphone spikes individually to a frequency as high as 45 per sec. In one experiment, however, spikes from a single unit were followed up to this frequency; it was found that they preserved their original size and individuality, and there was no rise in the base-line as there is in the usual mechanical record of a tetanus.

Simultaneous records of the electrical action potentials and of the 'sounds' can be obtained with the microphone shown in Fig. 1, which has a pair of electrodes centred in its orifice. Although it is a fairly simple matter to obtain a record from a single unit with a microphone alone or with a pair of electrodes alone, one has usually to make a number of trials before finding a place from which simultaneous electrical and microphone records may be made from the same unit: a loud-speaker on the output of the channel recording the action potentials is almost essential. Records of this kind are seen in Fig. 3, *b* and *c*. Here the activity of one particular unit is conspicuous, and it is clear that each action potential from this unit is accompanied by a 'sound'. This confirms the expectation put forward at the beginning of this paper.

The 'sounds' recorded by the microphone must be caused by minute movements in the motor units lying under its orifice. In the hope of investigating the nature of these movements more precisely, and of measuring the brief time interval between the electrical action potential and the movement itself, and also, possibly, of recording from a smaller area, we have used the piezo-electric system shown in Fig. 2. This instrument has the advantage of picking up both action potentials and mechanical movements from the same small area of contact with the skin. Even though this area is small, it is clear that both the electrical and the mechanical changes may be produced by units lying some distance away. Since the piezo-electric instrument records any movement of its point relative to the main body of the instrument, it was impossible to hold it sufficiently steady by hand. The head was therefore supported in a plaster rest to which the piezo-electric instrument was rigidly attached, and the subject tried to relax his neck muscles as completely as possible. Even then, the base-line of the records was never as steady as it was with the microphone. In other ways the records (see Fig. 3*d*) were similar to the microphone records, and showed the same range of frequencies of spikes and the same relationship to the action potential records. The spikes were monophasic, or more commonly diphasic, with a comparatively slow return to the base-line, the diphasic spikes having a total duration of 30–40 msec. The initial deflexion corresponded, as in the microphone records, to an outward movement of the surface of the eyelid.

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* This method was demonstrated at the XVII International Physiological Congress (Oxford, July 1947).

In order to measure the time interval between the electrical and the mechanical spikes, snapshots of single sweeps of the cathode-ray tube were taken at intervals of about 3 sec. In using this hit-and-miss method of making records, it was necessary to make sure before starting that the electrical and mechanical traces were from the same unit. The sweep was run as fast as was compatible with fitting a complete mechanical spike on to the face of the tube. It was found that the beginning of the electrical action potential preceded the beginning of the mechanical movement by an interval which lay, in different experiments, between 0·4 and 2 msec.; in any one experiment on one unit, the interval was consistent to about 0·5 msec. The results shown in Fig. 3*e* illustrate two extremely brief intervals. Even if the conditions of the experiment allowed the use of a faster sweep, there would be little or no increase in accuracy, since the point of rise of the mechanical spike is not sufficiently well defined.

DISCUSSION

The work described in this paper shows that an electrical action potential in a single motor unit is accompanied by a mechanical change which starts rapidly enough to produce a sound. The action potential and the brief mechanical change are physiologically related, and the correspondence between them was not due to any interaction between the electrical and mechanical recording systems: if it had been an artefact, there would always have been some correspondence, whereas, as previously explained, lengthy adjustment was usually necessary in order to achieve it.

Inspection of the spikes recorded with the microphone and with the piezo-electric instrument has shown some obvious similarities and some differences (compare (*b*) and (*d*) in Fig. 3). This calls for a closer analysis of the properties of these two instruments and of the response of each to muscular contractions.

A wave of muscular contraction cannot travel along the fibres comprising a single motor unit unaccompanied by a wave of lateral expansion. If the orifice of the microphone is placed on a region of skin under which there is an active motor unit, then, when the front edge of the travelling wave of lateral expansion reaches the region under the orifice, the skin will be forced to bulge slightly into the microphone chamber, thereby increasing the pressure in it and actuating the diaphragm. It is to be expected that an initial deflexion will occur when the sharp front edge enters the orifice, and a deflexion in the reverse direction when it leaves. This might explain the brief and diphasic spikes on the microphone record.

The charge on the crystal of the piezo-electric instrument is proportional, on the other hand, to the force exerted at any instant by the motor unit, except that it leaks away at a rate depending on the time constant of the input circuit (15 msec.). The sharp rise of the recorded spike occurs in a time considerably less than this time constant, and is therefore a fairly true representation of the force exerted on the pick-up point by the motor unit at any instant: it probably corresponds to the complete diphasic spike in a microphone record. Since the rest of the spike occurs more slowly, it is a less true picture of the contraction process: the apparatus does not give a faithful representation of the rise in mechanical base-line during a tetanus. It seems probable that the contracting motor unit exerts a force on the pick-up point not only by means of its lateral expansion, but also by means of its lengthwise contraction, because the pick-up point makes a dimple in the skin which the motor unit tends to flatten out when it contracts.

We have mentioned that the measured interval between the beginning of the electrical action potential and the beginning of the mechanical spike in a single motor unit varied considerably from one experiment to another. Although our figures indicate that the interval is a brief one, there may be a serious source of error in any measurements of this kind. It is known that synchronization of the discharges of different motor units can occur in powerful voluntary contraction of human limb muscles (Adrian, 1932, 1947), and from a few experiments we suspect that this occurs in m. orbicularis oculi even with moderate contraction. It is clear that the electrical and the mechanical records might, on occasion, be derived from different motor units which were discharging synchronously or nearly so, and if this were to happen the measurement of the interval between the electrical and mechanical spikes would be misleading.

Cooper & Eccles (1930), in a comparison of the twitch durations of different mammalian muscles, measured the stimulus frequency necessary to achieve an apparently smooth contraction. The frequency for m. gastrocnemius was 100 per sec., and for m. rectus internus 350 per sec. The palpebral part of m. orbicularis oculi, which is used for making extremely rapid movements, probably has a relatively high fusion frequency, and single units of this muscle could not achieve smooth contraction in the range of frequencies which we have observed in voluntary movements (less than 50 per sec.). It is doubtful, indeed, whether the contraction of single units even in a relatively slowly acting limb muscle is ever completely smooth, since Rutherford (1886) stimulated a whole muscle repetitively, and was able to hear a sound from the muscle whose frequency followed the stimulus frequency up to 352 cyc. per sec. These sounds were presumably produced in the way we have previously described, and were only observable from a whole muscle because all the motor units were being stimulated synchronously. The complex rumbling sound which is heard from large muscles during voluntary contraction must be due to the asynchronous activation of many motor units: the overall contraction of the muscle is then relatively smooth.

SUMMARY

1. The sounds heard by the human ear during the contraction of muscles adjacent to the meatus may be resolved into sets of rhythmic clicks, each set corresponding to the contraction of an individual motor unit. The sounds heard during the contraction of a single unit of m. auricularis superior have been recorded from a microphone inserted in the meatus. The optimal conditions for hearing these sounds, and the probable method of their conduction to the ear-drum, have been discussed.

2. The sounds produced by single motor units of m. orbicularis oculi were heard with a ‘stethoscope’ applied directly to the skin overlying the muscle, and were recorded by applying a microphone to the skin. In other experiments, the movements underlying these sounds were recorded through the skin with a piezo-electric instrument.

3. Each movement or sound in a series is associated with an electrical action potential which was recorded simultaneously from the same motor unit: the frequency of discharge varies with the strength of voluntary contraction, the highest recorded figure being 45 per sec.

4. Each movement lags behind its accompanying action potential by an interval of 2 msec. or less: the errors in making this measurement have been discussed.

5. The method by which a muscle achieves a smooth voluntary contraction is briefly discussed.

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THE MEASUREMENT OF CHLORIDE LOSSES IN THE SWEAT

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The classical studies of salt loss during sweating were based upon the total recovery of all the sweat produced (Hancock, Whitehouse & Haldane, 1929; McSwiney, 1934; Dill, Hall & Edwards, 1938; McCance, 1938). Although accurate this method is cumbersome and not generally applicable. The balance method, used by Henschel, Taylor & Keys (1943), is accurate but only useful for periods of 24 hr. or longer. Most authors have utilized a sampling method, the sweat samples being obtained, in some cases, by scraping (Moss, 1922; Weiner, 1936) but more commonly by putting an impermeable glove on one hand and allowing sweat to accumulate inside (Robinson, Dill, Wilson & Nielsen, 1941; Johnson, Pitts & Consolazio, 1944). Mickelsen & Keys (1943) reported, however, that the salt loss calculated from the salt content of glove sweat was 50 % higher than the actual salt loss measured directly.

This communication reports the results of direct measurements of salt losses during exercise in hot humid environments and compares these with the losses as calculated from the chloride content of sweat samples obtained over the same period from the whole arm, in distinction to the hand only.

METHODS

The subject was washed with distilled water until chloride free, dried and taken naked, except for the sweat bag on one arm, into a room maintained at a high temperature and humidity. The sweat bag, of artificial silk impregnated with a synthetic resin, was tied firmly round the arm 10–12 cm. below the tip of the acromion but was loose everywhere else; the hand, forearm and upper arm were thus totally enclosed and the sweat that collected was drawn off through a tube tied into the bottom of the bag.

The subject was weighed immediately on entering the room and at intervals thereafter; fluid intake and urine output were also measured. The duration of the exposure varied, but never exceeded 3 hr.; the time was spent in cycles of alternating rest and work (Ladell, 1947), but the whole time the subject remained in a bath, in the bottom of which was about 10 l. of distilled water. After each bout of work, and before and after each weighing, the subject wallowed in this water and thoroughly rinsed himself. Before weighing, he dried himself on a weighed towel for a measured period of time, ½–1 min.; any sweat that dripped off him while he was being weighed was collected in a drip-tray on the seat of the man-balance, and the drippings were later rinsed into the bath. Measured samples of bath water were taken at the beginning and end of each cycle, and the sweat bag was tapped twice in each cycle.

The volume of water in the bath at the time when each sample was taken was estimated by noting the height of water in a manometer tube connected to the bath through the plug-hole; the manometer was recalibrated for each experiment. This was found to be more satisfactory than estimating the volume by adding a known quantity of some solute and determining its dilution. In all cases the volume of water remaining in the bath at the end of the exposure was measured directly.

Chloride was estimated in the sweat by Whitehorn's method as for urine; and in the bath water by the same method using 100 c.c. portions and omitting the dilution with distilled water.

Calculation of results. The chloride content of the sweat from the whole body was calculated for each cycle from the amount of sweat lost, estimated from the weight change, urine output and fluid intake, and from the amount of chloride recovered; this consisted of the sum of the following: (1) The increase in chloride content of the bath water. (2) The chloride content of any bath water samples taken. (3) The chloride contained in the bath water absorbed by the towel (estimated from the weight increase of the towel, less the sweat lost from the body during drying). (4) Chloride content of the sweat lost from the body during drying (estimated from sweat rate and drying time). This item involves the assumption that the chloride content of the arm sweat is the same as that of the body sweat, but, as the amounts concerned are small, the error is negligible. (5) The chloride content of the samples from the bag.

The total chloride lost during the whole exposure was calculated separately from the direct and accurate measurement of the bath-water volume made at the end of the experiment.

Comparisons were made, for the whole exposure, between the total chloride recovered and the expected loss, calculated on the assumption that the chloride content of body sweat was the same as that of the bag samples. Comparisons were also made, for each cycle, between the observed concentration in the bag samples and the concentration in the whole body sweat, estimated as above.

RESULTS

Total loss for whole exposure

Sixteen experiments were done on seven different subjects. In thirteen the chloride recoveries were less than 6 g. (expressed as sodium chloride); in the other three, the subjects worked to exhaustion and stayed in the heat longer, losing more than 10 g. sodium chloride.

The mean estimated loss for the whole series was 5·67 g., and the mean recovery 5·55 g.; taking only the low losses, the mean estimated loss was 3·68 g. and the mean recovery 3·65 g., all as sodium chloride. Fig. 1 shows the estimated losses plotted against the recoveries and the regression line calculated for the full series; the correlation coefficient was 0·93 ($t=9{\cdot}47$) and the regression coefficient of recovery upon estimated loss 0·867. When only low losses were considered the respective coefficients were 0·99 ($t=23{\cdot}27$) and 1·082. The mean excess of the estimated loss over the recovery was, for the whole series, 0·128 g. and, for the low losses, 0·011 g.; the respective standard errors were 0·184 and 0·091, hence the differences did not differ significantly from zero, and for a whole exposure the actual chloride loss is the same as the loss estimated from the bag samples.

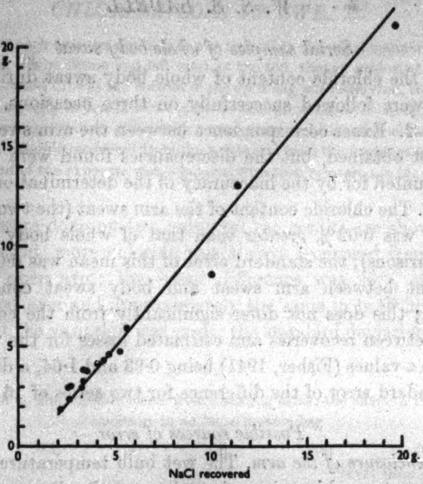

Fig. 1. Chloride losses in the sweat. The estimated losses have been plotted against the actual amount recovered; the correlation coefficient is 0·93, and the corresponding regression line is shown. (Chloride expressed as g. NaCl.)

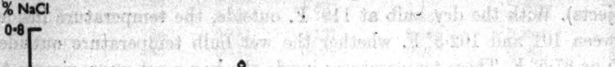

Fig. 2. The changes in the chloride concentration of arm sweat and of body sweat during the course of a single exposure. Solid circles, arm sweat; open circles, body sweat; dotted lines, subject L in dry bulb 100° F., wet bulb 94° F.; solid lines, subject L in dry bulb 104° F., wet bulb 94° F.; broken lines, subject H in dry bulb 104° F., wet bulb 93·5° F.

Serial samples of whole body sweat

Changes in the chloride content of whole body sweat during the course of an exposure were followed successfully on three occasions. The results are shown in Fig. 2. Exact correspondence between the arm sweat and the body sweat was not obtained, but the discrepancies found were no greater than could be accounted for by the inaccuracy of the determinations of the volume of bath water. The chloride content of the arm sweat (the two samples for the cycle pooled) was 0·02% greater than that of whole body sweat (mean of sixteen comparisons); the standard error of this mean was 0·025. The correlation coefficient between arm sweat and body sweat concentrations was 0·73 ($t=4{\cdot}01$); this does not differ significantly from the coefficient for the comparison between recoveries and estimated losses for the whole exposure, the respective z values (Fisher, 1941) being 0·93 and 1·64, a difference of 0·71, while the standard error of the difference for two series of 16 is 0·379.

Possible sources of error

From total enclosure of the arm. The wet bulb temperatures inside the bag were observed on two subjects in various climates. In all cases the temperature in the bag rose for the first 20 min. and then became steady. When the dry bulb temperature outside was 92° F. and the wet bulb outside 87° F. the wet bulb temperature inside the bag was 94–95° F. (three observations on two subjects). With the dry bulb at 119° F. outside, the temperature inside was between 101 and 102·3° F. whether the wet bulb temperature outside was 84·5 or 87·5° F. These temperatures inside the bag must approximate to the surface skin temperature inside the bag as there is no convection and the air is saturated.

Kuno (1934) quotes experiments which indicate that the rate of sweating may be affected locally by varying the temperature to which the given area of skin is exposed. If the composition of sweat is similarly affected, then enclosing an arm might alter the sweat composition also, especially in high dry bulb temperatures when conditions inside the bag differ considerably from those outside. The effect of enclosing an arm upon the total loss of chloride from that arm was therefore investigated.

First it was shown that, under similar conditions, the chloride losses are the same from both arms. On sixty-one occasions samples were collected from both arms simultaneously. The mean difference in chloride content between the two samples was 0·018% (S.E. ±0·003). Differences up to 0·092 were found, but the distribution was skew; in forty pairs the difference was less than 0·020%, and the g_1 statistic was 3·33 (S.E. of g_1 for sixty-one normally distributed observations is 0·31).

The chloride losses from an enclosed arm were then compared with those from the opposite arm left open to the atmosphere.

A bag was worn on each arm; on one side it was tied off in the usual manner, but on the other it was stretched over a wire frame and left open at the top, though protected from sweat drips from elsewhere. The outlet tube at the bottom of the open bag was connected, through a collecting bottle, with a suction pump in continuous action, so that all sweat went straight into the bottle, and the air inside the bag remained at the same temperature and humidity as the rest of the room. The total amount of chloride recovered from the bottle and from the washings from arm, bag and connexions at the end of the exposure was compared with that recovered in the samples from the opposite arm.

Eleven tests were done on two subjects; the results are shown in Table 1. With one exception, more chloride was always recovered from the enclosed than from the open arm; the mean difference was 26% of the chloride from the enclosed arm and approximately the same in both hot wet and hot dry climates, but the variation was great; the standard deviation for the whole series was 21%.

Table 1. The chloride losses from sweating arms: the effect of total enclosure in an impermeable bag

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		Sweat from enclosed arm			Sweat from open arm		
Subject	Climate	Vol. (c.c.)	Cl conc. (% NaCl)	Total Cl (g. NaCl)	Cl recovered (g. NaCl)	Estimated conc. Cl (% NaCl)	Estimated vol. (c.c.)
B	Hot wet	270	0·527	1·420	1·611	0·597	306
B	,,	248	0·505	1·252	1·157	0·467	229
B	,,	48	0·300	0·144	0·052	0·109	17
B	,,	36	0·381	0·137	0·085	0·236	22
H	,,	85	0·116	0·099	0·057	0·067	49
B	Hot dry	224	0·560	1·255	1·069	0·477	191
B	,,	187	0·516	0·965	0·832	0·445	163
B	,,	124	0·387	0·480	0·370	0·299	96
B	,,	159	0·545	0·870	0·561	0·353	103
H	,,	103	0·146	0·150	0·124	0·120	85
H	,,	195	0·252	0·491	0·308	0·158	133

The greater chloride losses from the enclosed arm could have been due either to a greater volume of sweat being excreted or to a greater chloride concentration in that sweat. In Table 1 are shown, in column 7, the original chloride concentration in sweat from the open arm calculated on the assumption that the same amount of sweat was produced on both sides, and in column 8 the original volume of sweat from the open arm calculated on the assumption that the chloride concentration was the same on both sides; thus allowance is made for any effect of evaporation. Except for the first experiment, the calculated figures for the open arm are less, both for chloride concentration and for volume, than the observed figures for the enclosed arm; thus sweat from an enclosed arm tends to be both more profuse and more concentrated than sweat from an open arm in both hot humid and hot dry climates.

From evaporation through the bag. Bags containing saline solution with the approximate concentration of sweat were hung up in the heat and samples were drawn off for analysis from time to time. No change in concentration was found even after 155 min. in air at 100° F. dry bulb and 95° F. wet bulb (R.H. 82%); but in 115 min. at 120° F. dry bulb, 80° F. wet bulb (R.H. 15%) the concentration increased in one experiment from 0·495 to 0·505%. In view of the fact that the sweat samples were never left longer than 20 min. before being taken out, evaporation, though it occurs, is too slow to give rise to error.

DISCUSSION

Mickelsen & Keys (1943) reported that the chloride content of sweat varied according to the part of the body from which it came. But the results reported here show that, in a hot humid climate, if the whole arm is enclosed, the sweat sample which can be collected has the same chloride content as the mixture of sweats from all parts of the body, and the total chloride losses from the sweating body may be calculated from the chloride concentration of the arm samples.

The sweat produced from a totally enclosed arm, however, is more profuse and contains more salt than that from an arm which is not enclosed. It may be this enclosure effect that, on the one hand, renders samples collected in arm bags valid samples of whole body sweat and on the other, gives rise to the gross error seen with glove samples. If the chloride concentration of hand sweat were to be initially high, and that of arm sweat low, then the enclosure effect would result in samples from glove collections having too high a chloride concentration, but it would bring the samples from bags with hand and arm sweat mixed to the average value for the whole body. This is what has been observed, and it suggests that the concentration distribution is the opposite to the intensity distribution described by Weiner (1945), the intensity on the arms being twice that on the hands.

The sampling method was not tested in hot dry climates as in high dry temperatures the bath water would have been too hot for the subject to rinse himself. But, as the enclosure effect was the same for both climates, hot wet and hot dry, sweat samples from enclosed arms are probably as good an indication of the whole body sweat in the one type of climate as in the other.

The bag method has been used in the field (Ladell, Waterlow & Hudson, 1944) and in laboratory investigations. The variation in the chloride concentration of sweat samples has been great, both between different individuals and for the same individual under different conditions. The mean chloride concentration in 187 samples of sweat from subject H was 0·275%, standard deviation 0·082; the mean concentration in 281 samples from subject B was 0·469%, standard deviation 0·091. The factors concerned in these variations are many (Johnson *et al.* 1944).

SUMMARY

1. The chloride content of whole body sweat is the same as that of sweat samples obtained from an arm totally enclosed in a bag.

2. This does not indicate that the chloride concentration in sweat is the same for all parts of the body, but is probably a chance effect due to the difference in concentration of sweat from different parts of the arm and hand, and to the modifying effect of total enclosure.

This work was done while working for the Medical Research Council at the Neurological Research Unit, National Hospital, Queen Square, London, W.C. 1, under the direction of Dr E. Arnold Carmichael, whom I thank for valuable advice. I am grateful to my fellow members of the Heat Physiology Team for their help and for their ready co-operation in allowing me the sole use of our combined facilities for this investigation. I also thank the various R.A.M.C. personnel who assisted me technically or as subjects, especially Staff-Sergeant W. Hammond, and Corporal C. M. Colquhoun.

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THE ACTIVITY OF THE DUODENUM

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This investigation of the activity of the duodenum was undertaken first to find out if the motor response, which appears in the jejunum, ileum and colon after feeding, also appears in the duodenum; and secondly to determine the frequency of the rhythmic contractions of the duodenum. Observations were made on trained dogs, in whom the duodenum had been exteriorized and enclosed in a tube of skin, a preparation originally applied to the small intestine by Biebl (1930), and later to the colon by Douglas & Mann (1940). It has the advantage over the Thiry and Thiry-Vella loops that, being in continuity, it is functionally active and takes its full share in the digestion and transport of food.

METHODS

Three suitably docile animals were selected, trained to lie on an observation table for periods of 2 hr. and to eat a standard meal of boiled dried meat, vegetables and biscuits in that position. Under general anaesthesia with intravenous pento-barbital and with full aseptic precautions, the duodenum was then exteriorized by the following operation.

Two parallel incisions were made in the abdominal wall immediately lateral to the mammary lines and the skin flap thus outlined was dissected up. The peritoneal cavity was opened in the mid-line and the duodenum delivered into the wound (Fig. 1A). After ligation of the branches reaching it from the superior and inferior pancreatico-duodenal vessels, the uncinate process of the pancreas was freed from the duodenum. The pancreas, as far as the main pancreatic duct, was then detached by blunt dissection and swung upwards to the greater curvature of the stomach (Fig. 1B). An incision was made in the mesentery of the duodenum, care being taken to avoid damage to the vessels. The pancreas, stomach and first part of the duodenum having been returned to the peritoneal cavity, the remainder of the duodenum was exteriorized by suturing the peritoneal and muscular layers of the abdominal wall through the gap in its mesentery (Fig. 1C). Experience has shown that at this stage it is necessary to divide the muscles for a distance of 1 cm. at right angles to their fibres on each side of the gaps through which the ends of the loop leave and re-enter the peritoneal cavity. If this is not done, stenosis of these openings eventually causes intestinal obstruction. Finally, the exteriorized gut is enclosed in the bi-pedicled skin flap already prepared (Fig. 1D).

After recovery from the operation, the animals remained in good health, and as in the case of other animals in which this technique has been used at lower levels of the intestine, they appear to suffer no inconvenience from the exteriorized duodenum. Indeed, one of the bitches whelped and successfully reared four pups after this operation, and 14 months later is still in excellent condition.

Tracings of the activity of the loop were taken by an air displacement system described elsewhere (Douglas & Mann, 1941). Information was also obtained by manual palpation and by inspection of the exteriorized viscus. Before an observation was made, the animal was fasted for 24 hr.; it was then placed on the observation table and a continuous tracing, lasting about 90 min., was taken before, during and after the administration of the standard meal.

The necessary ligature of some vessels probably interferes to some extent with the extrinsic nerve supply of the duodenum, but since the main blood supply is intact it is likely that this interference is minimal. Moreover, since the animals maintained a high standard of health it is likely that the exteriorized duodenum was functioning in a fairly normal manner.

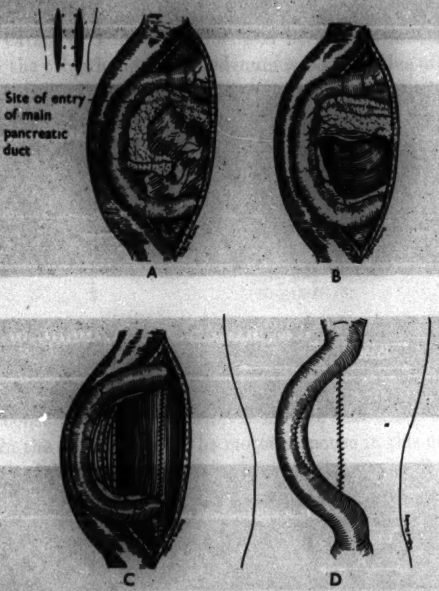

Fig. 1. Technique of preparation of an exteriorized loop of duodenum in continuity.

RESULTS

General character of duodenal motility. Tracings of duodenal motility resemble those from lower levels of the small intestine; the same long periods of quiescence are observed in the fasting animal, and the same motor response follows the ingestion of food (Fig. 2). However, regular contractions are more common in the duodenum than in the ileum, and consist of serial waves of contractions which pass rhythmically down the loop propelling the food towards the jejunum. These waves differ from the rhythmic segmentation seen in loops at lower levels in that contraction and relaxation of adjacent intestinal segments cannot be discerned. It may be noted that a similar type of propulsive activity in the human duodenum can be demonstrated by X-ray after the ingestion of an opaque meal; this suggests that, both in the human and in the canine duodenum, transport rather than trituration is the main function of the duodenal muscle coat.

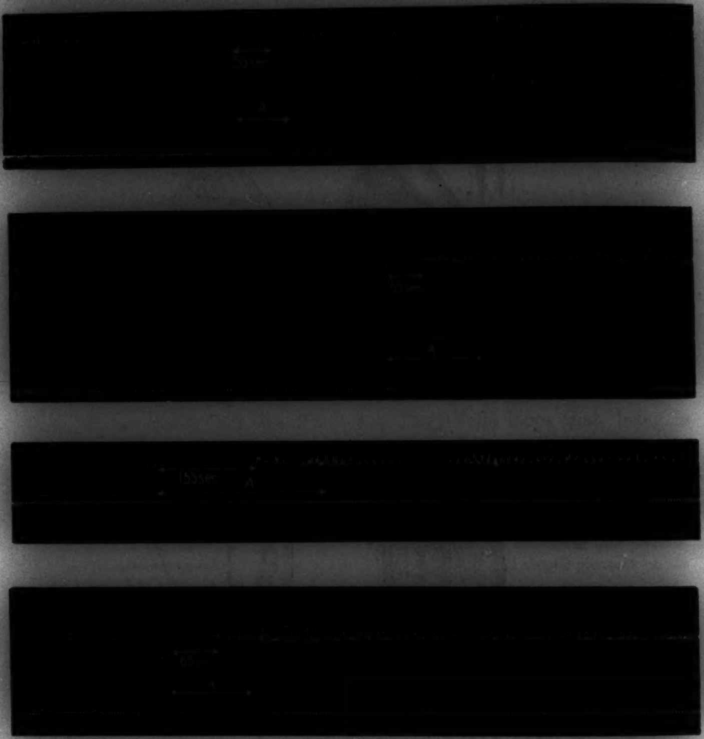

Fig. 2. Tracings of post-prandial excitation of the duodenum. Time in 5 sec. intervals. At *A* the animal is fed. In the upper two tracings records have been taken from the upper and lower ends of the loop simultaneously. The onset of the excitation varies from 55 to 155 sec. after the beginning of the meal.

Less than a second elapses between the appearance of the wave at the top of the loop and its disappearance at the lower end some 15 cm. distant; the wave thus travels between 15 and 30 cm./sec. This is much faster than the rate of peristaltic waves originally described for the small intestine of the dog by Bayliss & Starling (1901). They described a slow wave progressing down the intestine at the rate of 2 cm./min. The rate of 30 cm./sec. is much closer to that described by Alvarez (1924) in the small intestine of the rabbit, and termed by him the 'peristaltic rush'; he described peristaltic rushes with rates as fast as 25 cm./sec. Though antiperistalsis is commonly described in the human duodenum, evidence that it occurs in the dog was not obtained in this study.

The effect of the ingestion of food. If after a 24 hr. fast the duodenum was quiescent, the eating of a meal was followed by a rapid increase in activity (Fig. 2). If, on the other hand, the duodenum was still active after this period of fasting, no additional activity could be detected. The time of appearance of the motor response in the duodenum varied somewhat from animal to animal (Table 1). In dogs 1 and 3 the response appeared within 60 sec. of the beginning of the meal, whereas sometimes in dog 2 it did not appear for 175 sec. It may be noted in passing that dog 2 was rather more difficult to train than the other two animals; it was necessary to feed it by hand, and this resulted in a much slower rate of consumption of the meal. It may be that this is the explanation for the delay in the appearance of the motor response in this animal.

TABLE 1. Time from feeding to appearance of motor response in the exteriorized duodenum of the dog

Dog	Mean time in sec.
1	53 ± 5·8 (6)
2	138 ± 30·2 (5)
3	65 ± 13·1 (6)

The frequency of rhythmic contractions. In a previous study (Douglas & Mann, 1939) it has been found that in the jejunum and the ileum of the dog the frequency of rhythmic contractions is constant within narrow limits for any given segment of the intestine. In the present study the same was found to hold true for the duodenum (Table 2). In one dog the frequency of rhythmic contractions was between 17 and 19 contractions per min. in over 300 readings during a period of 17 consecutive weeks of observations, and the mean frequencies in the three dogs were 17·7, 17·9 and 17·3.

TABLE 2. The frequency of rhythmic contractions in the exteriorized duodenum of the dog

Dog	Mean number of rhythmic contractions per min.	Period of observation in weeks
1	17·7 ± 0·71 (336)	17
2	17·9 ± 0·67 (129)	7
3	17·3 ± 0·61 (69)	5

DISCUSSION

Though much work has been done on the activity of the first part of the duodenum proximal to the entry of the common bile duct, much less is known of the activity of the duodenum beyond that point. This is because it is difficult to prepare a Thiry-Vella loop at this level. If the animal were to be kept in reasonable health, it would be necessary to transplant both the common bile duct and the pancreatic duct.

From radiographic studies, the first part of the duodenum in man and in the dog appears to act in a different way from the remainder of the small intestine. Its movements are reasonably comparable to those of a chamber of the heart. It shows a period of diastole during which it receives barium from the pyloric antrum, succeeded by a period of systole during which the barium is ejected into the remaining portion of the duodenum. The frequency of this cycle does not appear to have been studied accurately. During the diastolic phase the characteristic 'duodenal cap' is seen. As the barium passes into the remaining portion of the duodenum, it is rapidly whisked round to the duodeno-jejunal flexure and radiologists agree as to the considerable speed of this wave. Segmentation is not normally observed radiographically in the human duodenum. Antiperistalsis in the human duodenum is said to be seen in the condition of duodenal ileus when the barium can be observed to pass round to the obstruction and subsequently to return to the duodenal bulb. It is doubtful if this appearance proves the existence of reverse peristalsis. It is easy to postulate that forcible propulsion of barium against an obstruction would result in its rebound in a reverse direction. However that may be, it is difficult to find reliable evidence that under normal conditions antiperistalsis takes place, and although particular attention was paid to the point in this study, evidence of its occurrence was not obtained. The rapid propulsive type of wave which appears to be the common type of activity in these loops is perhaps comparable with the whisking wave seen by radiologists in the human duodenum, and it is reasonable to suppose that its function is to propel the mixture of food and enzymes to the more absorptive reaches of the small intestine.

The appearance in the duodenum of a motor response to feeding is of interest since a similar response has been observed at other levels of the small intestine and colon (Douglas & Mann, 1940). It provides further evidence that a wave of activity passes down the whole length of the bowel when food is first taken after a fast. Its appearance in the ileum has been termed by Hurst (1913) the gastro-ileal reflex, and in the colon the gastro-colic reflex. It is doubtful, however, whether such terms are justified since the reflex is not abolished by section of the extrinsic nerves of the intestine, and the waves can traverse a recent anastomosis. The time of the appearance of the response in the duodenum is very similar to that of its appearance in the jejunum where in two animals it appeared 72 and 84 sec. from the beginning of the meal. In all likelihood the function of this post-prandial wave is to empty the intestine before more food arrives from the stomach. When the wave reaches the rectum, defaecation may take place. Thus the function may be an excretory one, analogous to the act of micturition; but whereas in the latter the stretching of the muscle fibres in the wall of the bladder is the adequate stimulus, in the former it is possibly the stretching of the muscle fibres in the stomach. It is difficult to find a suitable term for this physiological phenomenon, but 'post-prandial excitation of the intestine' seems to be the most suitable.

The uniform frequency of the rhythmic contractions in the duodenum is similar to that in the remaining portion of the small intestine. It should be emphasized that the constancy relates only to frequency and not to amplitude of contraction. On many occasions contractions are absent altogether and when they are present great variations in amplitude can be seen (Fig. 3). The explanation for this observation is difficult to find. It may be suggested that the frequency of rhythmic contractions is fundamental to the caudal progress of food residues as suggested by Alvarez (1915). There is no doubt that the frequency of rhythmic contractions falls at successive levels of the small intestine (Table 3). It will be noted from this table, which has been prepared

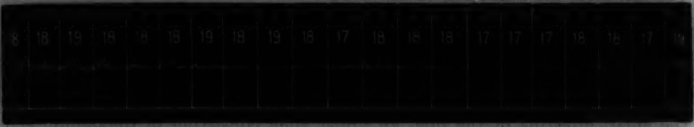

Fig. 3. Tracings of the activity of the duodenum of the dog. Time in 5 sec. intervals. Tracing ruled off at 1 min. intervals and number of contractions entered. Note that the amplitude of contractions varies while the frequency remains constant.

TABLE 3. Frequency of rhythmic contractions in the small intestine of the dog

Dog	Site of loop	Mean frequency of rhythmic contractions per min.
1	Duodenum	17·7±0·71 (336)
2	Duodenum	17·9±0·67 (129)
3	Duodenum	17·3±0·61 (69)
4	Jejunum, 15 cm. from lig. of Treitz	18·0*
5	Jejunum, 30 cm. from lig. of Treitz	17·6±0·77 (45)
6	Jejunum, 30 cm. from lig. of Treitz	17·1±0·93 (46)
7	Ileum, 75 cm. above caecum	13·9±0·72 (118)
8	Ileum, 60 cm. above caecum	13·8*
9	Ileum, 30 cm. above caecum	11·8±0·82 (58)
10	Ileum, 25 cm. above caecum	13·4±0·71 (107)
11	Ileum, 10 cm. above caecum	12·5*

* Necessary detailed data to calculate the standard deviation are not available for these animals in which the observations were made in 1938–9. In the recollection of the writer, however, the readings did not vary more than in the present study.

from the present study and from previous work, that there appear to be two distinct zones of rhythmic frequency in the small intestine of the dog, an upper zone in which the rate is between 17 and 19 contractions per min., and a lower zone in which it is between 11 and 14 contractions per min.

SUMMARY

1. The duodenum below the entrance of the common bile duct has been exteriorized in continuity in the dog.

2. A study of its motility has been made visually, by palpation and by mechanical recording.

3. The common type of activity observed was a rapid propulsive wave of a uniform frequency of between 17 and 19 per min.

4. Fasting usually produced quiescence of the loop, and subsequent feeding a rapid motor response. The onset of this response varied between 50 and 140 sec. after the beginning of the meal.

5. This evidence, together with evidence presented in previous publications, is taken to suggest that (*a*) after the taking of food in the fasting animal a wave of excitation passes down the whole length of the small intestine from duodenum to ileum, and (*b*) there appear to be two zones of rhythmic frequency in the small intestine of the dog, an upper zone consisting of duodenum and jejunum in which the frequency is of the order of 17 to 19 contractions per min. and a lower zone in which the frequency is between 11 and 14 contractions per min.

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OXYGEN TENSION AND THE RESPIRATION OF RESTING FROG'S MUSCLE

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Warburg, starting in 1908, was the first to investigate the effect of oxygen tension (p_{O_2}) on cellular respiration (Q_{O_2}) under conditions of optimal gas diffusion. He measured the respiration of sea-urchin eggs at 20° C. and found that the oxygen uptake was the same at tensions of 33 mm. Hg and 132 mm. Hg. The rate of oxygen uptake of nucleated red cells proved, at 0° C., to be unchanged at oxygen tensions varying between 5 and 75 mm. Hg. With *Micrococcus candicans* at 1° C. the rate of respiration even at 10^{-5} atmosphere partial pressure was the same as in air (Warburg & Kubowitz, 1931). Thus it came to be accepted as an axiom that cellular respiration is independent of variations in oxygen tension. Meyerhof (1930), discussing the calculation of the amount of oxygen necessary for the supply of the isolated muscle, writes: 'In the given formulae it is assumed that the rate of respiration of cells is independent of oxygen tension. This assumption corresponds to the general experience of the respiration of animal organs.'

Many of the exceptions that have been found to this 'all-or-nothing' law of cellular respiration have been criticized on the grounds of insufficient oxygen diffusion. The first real indication of a variation with tension came when it was shown that even at relatively high oxygen tensions the respiration of certain bacteria and of sarcoma cells appeared to be dependent on oxygen tension, and Kempner (1937, 1939) claimed that if one works at higher temperatures (25–42° C.), and with uninjured cells in a natural medium, there may be a marked decrease in respiration of certain blood cells and bacteria if the oxygen tension is lowered to 30–40 mm. Hg partial pressure. For example, at 37° C. the respiration of *M. candicans*, found by Warburg to be unchanged even at exceedingly low tensions of oxygen when he worked at 1° C., was greatly affected by lowering the oxygen tension; at 15 mm. Hg the rate of respiration was down to 40% of its full value.

Kempner used the Warburg manometric technique. More recently Winzler (1941) has studied the respiration of cell suspensions with the dropping mercury electrode, and failed to confirm some of Kempner's findings. He compared the results by the Warburg method with those obtained with a dropping mercury electrode, showing that the Q_{O_2} was actually independent of p_{O_2} to a very much lower value than would appear from the manometric method, even with the most dilute of the suspensions.

Winzler also found that at 20° C. the rate of respiration of yeast begins to decrease at a p_{O_2} of about 2·5 mm. Hg, falling to 50 % of normal at 0·5 mm. Hg. The 'critical' oxygen tension changes with temperature, being 0·6 mm. at 5° C. and 6·0 mm. at 34° C., and the rates fall to 50 % of normal at about 0·1 mm. at 5° C. and 1·7 mm. at 34° C. The above figures are for yeast suspension with 0·1 M glucose added. Without substrate, the rate of respiration is reduced, and the critical p_{O_2} is then lower. At 20° C., without substrate, the rate is unchanged until the tension falls below 0·2 mm. Hg. At 5° C. the critical tension would probably be about 0·05 mm. Hg. These figures give the best indication that is available as to what might be found in muscle with its similar metabolic system, and it is the purpose of this research to find out to what extent the prevailing assumption with regard to muscle can be substantiated.

In the literature there are no references to any study of the p_{O_2}-Q_{O_2} relation in muscle. In this tissue, diffusion becomes the governing factor. For instance, working on the assumption that Q_{O_2} is independent of p_{O_2}, the sartorius of a frog, 0·7 mm. thick exposed on both sides, would be fully supplied with oxygen at tensions not lower than about 24 mm. Hg at 20° C., and clearly there would be difficulty in examining the behaviour at any lower p_{O_2} by a standard gasometric method. Smaller muscles from the frog might be used, but these are generally spindle-shaped and not much thinner than the sartorius. Sheets of muscle such as the sterno-cutaneous are available, but the oxygen consumption of such a muscle at rest is only 0·001 cu.mm./min., and no manometer or volumeter would have sufficient sensitivity unless it were much smaller than it could be if a muscle such as the sterno-cutaneous had to be supported in it. Other thin muscles, such as those of the abdominal wall, are not suitable for a gas-exchange method because they cannot be freed from neighbouring tissues.

There were three ways of approach which seemed promising and which, between them, have contributed to the solution of the problem. The first was used for investigating the p_{O_2}-Q_{O_2} relation over a wide range of oxygen tensions to get the general picture, the other two for a closer study at low p_{O_2}. In each case the oxygen tension was measured by a platinum electrode.

METHODS

A brief description of the three methods comes first, followed by details of each in turn.

Method I. The general p_{O_2}-Q_{O_2} *relation*

A thin layer of frog's abdominal muscle, 100–300 μ. thick, is stretched over a glass plate in a bath of Ringer's solution and a second parallel plate placed on top of it, leaving a space of 100–200 μ. above the muscle. An oxygen-measuring device known as the 'flow electrode' is inserted in this space, and withdraws the fluid at a very slow rate, continuously measuring the oxygen tension in it. The muscle uses the dissolved oxygen, and the oxygen tension falls at a rate that is proportional to the respiration rate. Thus, in a single run, a record is obtained of the complete p_{O_2}-Q_{O_2} relation. Allowance has to be made for diffusion.

This method proved to be unreliable at low oxygen tensions. Its main purpose has been to demonstrate that the rate of oxygen consumption is independent of oxygen tension until the latter approaches to within a few mm. Hg above zero. Failure to give reliable results at low p_{O_2} is attributed to the system not being in a steady state. Errors may arise for the following reasons: (*a*) by gradients in the p_{O_2} between different parts of the muscle, due first to a possible non-uniformity in the thickness of the muscle in the area between the plates, and also by diffusion of oxygen from the edges of the plates towards the centre; and (*b*) by the presence of oxygen carriers (haemoglobin or myoglobin) in the muscle. If these are present in significant amounts a change in electrode current is not proportional to the respiration rate in the p_{O_2} range corresponding to the dissociation of the oxygen carrier.

Method II. The p_{O_2}-Q_{O_2} *relation at low* p_{O_2}

The sources of error inherent in method I are avoided by working on a steady-state principle, as follows. If one side of a sheet of muscle is exposed to a known oxygen tension, and the other side is bounded by an impermeable wall there will be a difference in p_{O_2} between the two sides of the muscle which is proportional to the rate of oxygen consumption of the muscle. If the p_{O_2} on the exposed surface of the muscle is progressively reduced, the p_{O_2} measured by an oxygen electrode at the impermeable boundary will decline in parallel as long as the Q_{O_2} remains constant. When the p_{O_2} at the impermeable boundary reaches the 'critical' value the difference between the inside and the outside will start to decrease. The electrode, being situated at the impermeable boundary, measures the minimum p_{O_2} in the muscle, and there can be no doubt as to the value of the p_{O_2} at which the respiration rate starts to diminish. This method, working on the steady state, could of course be used for studying the general p_{O_2}-Q_{O_2} relation as well. It was not developed, however, until method I was found to be inadequate for the accurate measurement at low p_{O_2}, and then there was no need to pursue the study of the p_{O_2}-Q_{O_2} relation above about 5–10 mm. Hg.

Method III

As with method II, the system is in a steady state. The arrangement of the muscle between glass plates is the same as in method I. The muscle is covered with the upper glass plate, and the system is left undisturbed for a period of about 3 hr. until a steady state is reached. The inner portions of the muscle have by then become anaerobic, but there is an outer fringe near the edges of the glass plates in which the muscle can be supplied with oxygen by diffusion from the outside. There is a gradient in p_{O_2} from the edge, where the p_{O_2} is equal to that in the external fluid in the bath, to zero as the anaerobic zone is approached. With air-saturated solution externally the width of the aerobic fringe is about 3 mm., and the gradient is 'mapped' by a micro-electrode of the static type, which can be moved to and fro by a micrometer screw in the fluid space above the muscle. The p_{O_2}-Q_{O_2} relation can then be deduced. So long as Q_{O_2} is independent of p_{O_2} the latter should fall parabolically as the electrode is moved in by equal steps from the outside, and a deviation (if any) from the parabolic curve would indicate the value of the 'critical' p_{O_2}. This method gives surprisingly high accuracy, particularly at low partial pressures, but the results are not quite comparable with those obtained by the other methods owing to the presence of lactic acid which diffuses out from the anaerobic zone. This point will be discussed.

Method I

A platinum electrode immersed in a conducting electrolyte solution and maintained at a negative potential relative to the bulk of the solution will reduce molecular oxygen to hydrogen peroxide ($O_2+2H^++2e=H_2O_2$) and by a further stage to water ($O_2+4H^++4e=2H_2O$). The current which passes between the platinum electrode and an indifferent non-polarizable anode elsewhere in the solution is proportional to the oxygen tension in the fluid (Davies & Brink, 1942).

An unusual form of the electrode was used in method I. For many purposes this 'flow' electrode is not so satisfactory as the ordinary 'static' type used in methods II and III, and needs more care and attention. It was used here, however, because no allowance need then be made for the oxygen consumed by the electrode itself. For reasons given later, there was no longer any difficulty in making allowance for the oxygen used by the electrode in method II, once the general p_{O_2}-Q_{O_2} relation had been established, and the ordinary type of electrode could then be used.

Design and use of the flow electrode (Fig. 1). A glass tube with very thin walls and of internal diameter about 2 mm. is drawn out in a micro-flame to a diameter of about 50–100 μ. A 60 μ. platinum wire, insulated in a glass tube to within 2 cm. of the end, is inserted into the lumen of the capillary extension, and pushed down until the glass insulating sheath is stopped by the constriction. The 2 cm. of exposed platinum wire constitutes the cathode and a 100 μ. silver wire, coated with chloride, acts as the anode. The open tip of the capillary tube is immersed in the Ringer's solution at a point close to the muscle. The solution is drawn up the fine tube at a constant rate by a negative pressure maintained at the wide end of the tube. As it passes the platinum wire it delivers up its oxygen, which is reduced, and the current which flows is proportional to the oxygen tension.

Current measurement. The current passed by the flow electrode varied from 10^{-6} to 10^{-9} amp. or down to 10^{-11} amp. in the case of the 'static' type electrode. The method chosen for amplification (Fig. 2) was to include a high resistance R of 0·1–100 MΩ. (varying with the sensitivity required) in series with the oxygen electrode, and to measure the voltage across the resistance by means of a cathode follower.

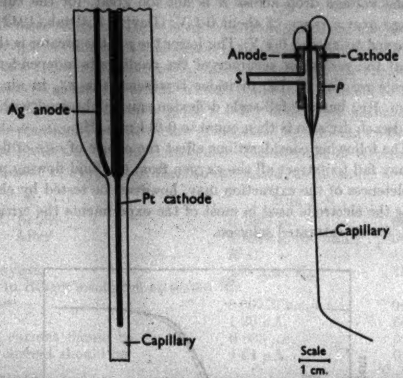

Fig. 1. The 'flow electrode'. The entire electrode, to scale, is shown on the right. The end of the capillary is shown on the left (not to scale). External diameter of capillary 50–100 μ. The 60 μ. platinum wire is insulated in glass to the point at which the wire enters the capillary. A 2 cm. length of free platinum wire lies in the capillary. The silver anode is not sheathed in glass. The glass tube with its capillary extension is mounted in a hollow perspex holder P, and a side tube S connects with the negative pressure system.

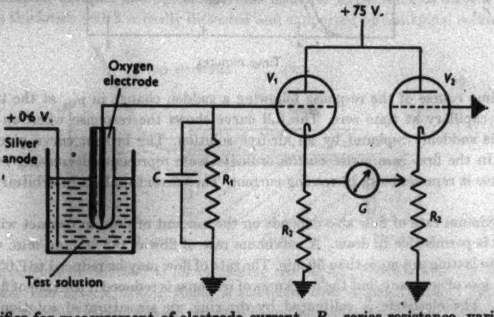

Fig. 2. Amplifier for measurement of electrode current. R_1, series resistance, variable from 0·1 to 100 MΩ. C, smoothing condenser (variable, depending on R_1, to give time constant of about 1 sec.). The voltage across the series resistance is applied to the grid of valve V_1. Valve V_2 is for balancing the effect of drifting anode voltage and its grid is earthed. The Cambridge 'Spot' galvanometer G is connected between the cathodes with a suitable balancing adjustment. The cathodes are earthed through resistances R_2 (20,000 Ω). The valves are pentode *RCA* 6C6 run as triodes at 75 V. The sensitivity of the galvanometer (450 Ω) is 170 mm./μA. when critically damped.

The driving voltage is applied across the electrode and across *R* in series. As a consequence of this the voltage across the electrode will vary with the p_{O_2}. This will not affect the electrode current so long as the voltage drop across *R* is not too great, for the current is very nearly independent of voltage over a range of about 0·4 V. (Davies & Brink, 1942). The voltage drop across *R* was not allowed to exceed 0·1 V. The lower the p_{O_2} the greater is the permissible value of *R*. This means that the *percentage* accuracy of the readings is independent of the p_{O_2}. For example, if a full-scale reading of 700 divisions represents the p_{O_2} in air (155 mm. Hg) the sensitivity is 0·22 mm. Hg; but the full-scale deflexion can be obtained with 10 mm. Hg if *R* is suitably adjusted and each division is then equal to 0·014 mm. Hg.

The rate of flow. The following considerations affect the choice of rate of flow:

(*a*) The cathode may fail to extract all the oxygen from the fluid flowing past it if the flow is too great. The completeness of the extraction may, however, be tested by checking the voltage-current relation. For the electrode used in most of the experiments the critical rate of flow was about 1 cu.mm./min. for air-saturated solution.

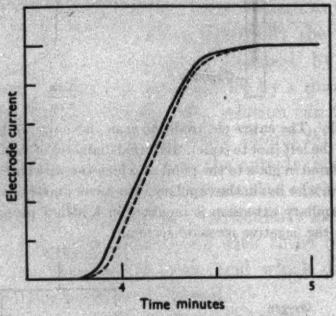

Fig. 3. The time course of the response following a sudden change in p_{O_2} at the tip of the flow electrode capillary at time zero. The full curve shows the response when an air-saturated solution is suddenly replaced by an air-free solution. The broken curve gives the reverse change. In the first case a rise on the ordinate scale represents decreasing current, in the second case it represents an increasing current. The current scale is in arbitrary units.

(*b*) The maximum rate of flow also depends on the amount of fluid in contact with the muscle upon which it is permissible to draw. A maximum rate of flow of 0·2 cu.mm./min. was judged to be safe for a run lasting not more than 50 min. The rate of flow may be reduced to 0·05 cu.mm./min. without much loss of accuracy, but the quickness of response is reduced if the rate of flow is lowered.

Calibration. The electrode is calibrated by drawing up air-saturated solution at a known temperature and barometric pressure.

Time course of response. There is a lag in the response to a change in oxygen tension which is due to the time taken for the fluid to pass up the capillary to the platinum wire. In the electrode used for most of the experiments recorded here the capillary was about 10 cm. long (unnecessarily long for some purposes, but it permitted immersion in a deep bath) and the time lag was about 4 min., for a rate of flow of 0·2 cu.mm./min. When the 'front' arrives at the cathode, the galvanometer does not immediately take up the new equilibrium reading owing to some mixing and diffusion in the passage up the tube. The necessary calibration is made by inserting the end of the capillary into a vessel in which the fluid can be changed rapidly. The response following a sudden change takes place as in Fig. 3. If the voltage between the tip of the platinum and the solution is less than that required for reduction of oxygen at that point, the time course of the response will depend on the actual value of the oxygen tension. In this event the time course of the reponse, following a sudden change from one p_{O_2} to another, is not identical with the reverse change, but the difference is small.

The nature of the electrode reaction. There is some uncertainty about the nature of the reaction which occurs when oxygen is reduced at a platinum cathode. Laitinen & Kolthoff (1941) give two arguments in favour of a 2-electron reaction ($O_2 + 2H^+ + 2e = H_2O_2$). On the other hand, Davies & Brink (1942) have observed electrode currents which appear to correspond to a 4-electron reaction ($O_2 + 4H^+ + 4e = 2H_2O$). The flow electrode gives a ready and direct means of investigating the nature of the electrode reaction. The rate of flow is measured by drawing up fluid for a known time from a weighed vessel, and the current is recorded. The number of electrons required for the reduction of each molecule of oxygen can then be calculated, as follows:

	Run I	Run II
Weight of test vessel: Before	5·1667 g.	5·1081 g.
After	5·1340 g. (60 min.)	5·0808 g. (95 min.)
Temperature	8° C.	14° C.
Partial pressure of oxygen	156 mm. Hg	152 mm. Hg
Solubility of oxygen in Ringer's solution at stated tension and pressure	0·00775 c.c./c.c.	0·0068 c.c./c.c.
Electrode current	1·20 μA.	0·54 μA.
If reduction to H_2O_2 current should be	0·605 μA.	0·28 μA.
If reduction to H_2O current should be	1·21 μA.	0·56 μA.

It therefore appears that the 4-electron reaction is taking place. The differences between measured and calculated currents must not be taken to indicate a real deviation from the 4-electron reaction; the experimental errors were probably enough to account for these differences.

Procedure

Dissection and preparation of the muscle. The body wall consists of three muscles, viz. the internal and external oblique, and the transversus muscles. These muscles form a thin layer of nearly uniform thickness which is easily dissected and supported by the spinal column, the pelvic bone and the xiphisternum. In a female frog, a 3 cm. square of uniform muscle tissue can be prepared. The thickness is variable; in a female it is 150–200 μ., in a male 200–350 μ. If a still thinner preparation is required, the external oblique muscle can be dissected off, leaving a sheet about 100 μ. thick in a female.

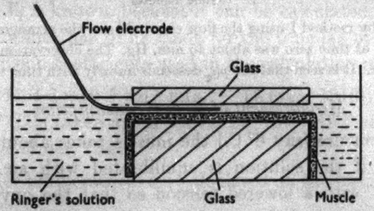

Fig. 4. The arrangement (method I) for measuring the p_{O_2}-Q_{O_2} relation with the flow electrode. *Not to scale.* The muscle, 250 μ. thick, is stretched over a glass plate, 2 cm. square. The thickness of the fluid space between the muscle and the upper glass plate is 150 μ. The outside diameter of the electrode capillary is 80 μ.

The blood is pressed out as far as possible, and the muscle is set up on clamps on a Palmer rack stand. It is then lowered and stretched over a 2 cm. square glass plate in a bath of Ringer's solution (Fig. 4). A second plate of glass of the same dimensions can be raised and lowered over the first by means of a micrometer screw so that the space between the two surfaces is known. The thickness of the muscle can also be measured with the same device. A layer of Ringer's solution, 100–200 μ. deep above the muscle, accommodates the end of the capillary of the flow electrode. The fluid in this layer is drawn up the tube, and its oxygen tension is measured continuously in the manner described.

The muscle is soaked for some hours before use to allow the respiration rate to settle to a constant value. Buffer solutions of phosphates at neutral pH could not be used because they had a deleterious effect on the electrode, the cause of which is not known, resulting in a progressive fall in the current for a given p_{O_2}, which was only slowly reversed on returning to normal solution. Phosphates at pH 6 did not act in this way.

Experiments were generally run at room temperature, but a few were done at higher temperatures, up to 30° C. For these a gas jet was placed under the bath, and the temperature controlled by hand to a few tenths of a degree.

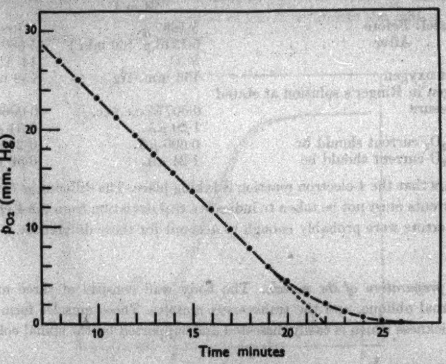

Fig. 5. Record taken by method I using the flow electrode and the arrangement shown in Fig. 4. 9·5° C. Initial p_{O_2} at time zero was about 45 mm. Hg. The observations, shown as dots, were made each minute. It is seen that the p_{O_2} descends linearly with time until about 5 mm. Hg.

Measurement of oxygen consumption

At a low temperature (say 6° C.) the muscle takes about 3 hr. to use the dissolved oxygen if the solution is equilibrated with air. It is therefore necessary to start with a lowered tension so that the oxygen is exhausted within reasonable time and the run does not last too long. 30 min. was the maximum allowed. With the upper plate raised (see Fig. 4), the muscle is flooded with Ringer's solution of the required oxygen content; the plate is then lowered and the readings commenced. The muscle uses the dissolved oxygen, the tension falls and the galvanometer readings are taken at intervals varying from 15 sec. to 1 min. (depending on the rate of respiration). A record obtained in this manner is shown in Fig. 5.

Analysis of results. The diffusion problem.

The rate of oxygen consumption is plainly independent of tension over a considerable range, and the object is to find the value of the 'critical' p_{O_2} at which this ceases to be so. The muscle does not fill the entire space between the plates and allowance has therefore to be made for diffusion. The lowest p_{O_2} in the system is in the part of the muscle which is farthest from the fluid space and the deviation from the linear p_{O_2}-time relation (Fig. 5) takes place when the p_{O_2} in this deepest layer of muscle falls to the 'critical' value. The electrode measures the p_{O_2} in the fluid space, and the corresponding p_{O_2} in the deepest layer of muscle has therefore to be calculated. After the initial 'settling down' the oxygen tension at any point between the plates falls in a linear manner at a rate $\frac{V_1 a}{T(a+b)}$, where a = thickness of muscle, b = thickness of fluid space, T = time taken for the muscle to use the quantity of oxygen dissolved in its own volume at a tension V_1. The solubility of oxygen in muscle is the same as in Ringer's solution.

The electrode measures a quantity which must approximate the *average* tension in the fluid space, and so long as Q_{O_2} is independent of p_{O_2} it can be shown that this exceeds the tension in the lowest layer of muscle by an amount

$$V_2 = \frac{V_1 ab}{T(a+b)}\left(\frac{a}{2k_1}+\frac{b}{3k_2}\right),$$

where k_1 is the diffusion coefficient of oxygen in muscle, and k_2 is the diffusion coefficient of oxygen in Ringer's solution.

When the oxygen tension in the lowest layer of muscle falls to the critical level V_3 at which respiration starts to decline with tension there is a deviation from the linear p_{O_2}-time relation. At that moment the electrode will show a tension V_3+V_2. V_2 is known, because it can be calculated, and V_3 is deduced accordingly. Allowance has to be made for the time lag of the electrode. The values of the diffusion coefficients used in the calculation of V_2 are:

k_1 (muscle) 20° C. $4\cdot5\times10^{-4}$ cm.^{2} min.$^{-1}$ (Krogh, 1918);
k_2 (Ringer) 20° C. is taken as for water, i.e. $12\cdot5\times10^{-4}$ cm.^{2} min.$^{-1}$ (Kolthoff & Miller, 1941).

The values at other temperatures are calculated on the basis of a 2·5% change per 1° C.

In measuring up the records the deviation from linearity is assumed to occur when the galvanometer deflexion is 5% higher than the continuation of the straight line.

The use of a 'static' type of electrode sealed in one of the glass plates would allow the plates to be applied closely to the muscle on both sides, and the above diffusion correction would then be avoided. But the problem of allowing for the oxygen consumption of the 'static' electrode proved to be difficult, and it was thought better, on balance, to use the flow electrode.

The results are shown in Table 1. The values of V_3 range between zero and 8 mm. Hg. Possible causes contributing to this variability have been mentioned above. Methods II and III should give more accurate results at low p_{O_2}.

TABLE 1.

Temperature (° C.)	T min. for $V_1=155$ mm. Hg	V_2+V_3 (mm. Hg)	V_2 (mm. Hg)	V_3 (mm. Hg)
6	133	1·8	0·48	1·32
7·5	92·5	7·0	0·7	6·3
7·2	140	4·4	0·4	4·0
6·3	152	1·8	0·4	1·4
6·3	145	5·4	0·4	5·0
9·2	56	4·0	0·9	3·1
9·5	40	5·9	1·4	4·5
9·5	54	4·2	1·0	3·2
9·8	20	3·4	2·8	0·6
9·3	20	5·0	4·5	0·5
8·9	20	3·3	2·8	0·5
8·9	20	3·5	2·8	0·7
8·8	20	5·0	4·5	0·5
8·8	20	3·3	2·8	0·5
8·8	20	6·8	5·9	0·9
9·0	21	6·8	2·8	4·0
9·1	21	6·9	2·8	4·0
9·0	21	8·5	2·8	5·7
9·0	21	10·2	2·8	7·4
15·0	11·4	14·8	6·9	7·9
15·0	14·2	12·5	5·8	6·7
15·0	15·5	11·2	5·3	5·9
15·0	15·3	10·0	5·3	4·7
30·0	7·2	10·0	5·5	4·5
31·0	6·2	5·0	6·2	—
30·7	6·2	8·5	6·2	2·3

V_3 represents the 'critical' p_{O_2} below which the Q_{O_2} appears to diminish with p_{O_2}.

Method II

A general description of the method has been given above. The experimental set-up is shown in Fig. 6. In Fig. 6 (*a*) the flow electrode is used, in Fig. 6 (*b*) the electrode is of the conventional 'static' type, consisting of a 50 μ. platinum wire sealed in the glass and ground flush with the surface. A mixture of nitrogen and oxygen is played over the upper surface of the muscle; when a steady state has been reached the p_{O_2} under the muscle is recorded by the electrode.

If V_2 is the oxygen tension in the gas mixture, V_3 the oxygen tension under the muscle, k the diffusion coefficient of oxygen in the muscle, a the thickness of the muscle, then the rate of respiration is given by

$$\frac{2k\,(V_2-V_3)}{a^2}.$$

Now $2k/a^2$ is constant, so that the respiration rate is proportional to the difference in p_{O_2} on the two sides of the muscle. The oxygen content of the gas mixture can be altered at will, and the respiration rate related to the lowest p_{O_2} in the muscle V_3. The muscle requires a p_{O_2} difference of about 5 mm. Hg to supply it with oxygen. It is this difference V_2-V_3 that is measured and found to be independent of V_3 so long as Q_{O_2} is independent of p_{O_2}, but which will diminish when V_3 becomes small enough and reaches the 'critical' value. The 'critical' p_{O_2} may be too small to measure, in which event V_3 will fall strictly in parallel with V_2 until it reaches zero, and then part of the muscle becomes anaerobic if V_2 is still further reduced.

If the flow electrode is used there is no correction to be made for oxygen used by the electrode; the amount of fluid that has to be withdrawn from the space between the muscle and the glass is insignificant. But if the 'static' type of electrode is used, a correction must be made for the oxygen consumed by the electrode itself. Part of the observed p_{O_2} difference between the two sides of the muscle is required for the transport of oxygen for the electrode reaction. The correction for this is easily made when the p_{O_2}-Q_{O_2} relation for some portion of the p_{O_2} range is established. The part of the p_{O_2} difference which is due to the electrode reaction is proportional to V_3, say fV_3, and the correction consists of subtracting this quantity from the actual difference. It is not possible to calculate the factor f from first principles with sufficient accuracy, but its value is found experimentally by taking account of the established fact (method I) that the Q_{O_2} of the muscle is independent of p_{O_2} above (say) 10 mm. Hg. A value of f is selected such that the corrected value of $V_1 - V_2$ is constant down to 10 mm. Hg. This procedure worked well and it was then no longer necessary to use the flow electrode, and the static type, which is easier to handle, could take its place. The factor was about 0·2 for the flush 50 μ. platinum electrode shown in Fig. 6 (*b*).

The muscle must be removed from the electrode when the exact composition of the gas mixture is measured, and it is then necessary that the gas should play upon a film or layer of unstirred fluid of definite thickness. For this reason a layer of silk (about 150 μ. thickness, close-woven) is interposed between the surface of the 'static' type electrode and the muscle. The combined thickness of silk and muscle must not be too great or the system becomes unduly sluggish in its response to a change in p_{O_2}. The time taken is about 4 min. for 150 μ. of silk plus the muscle.

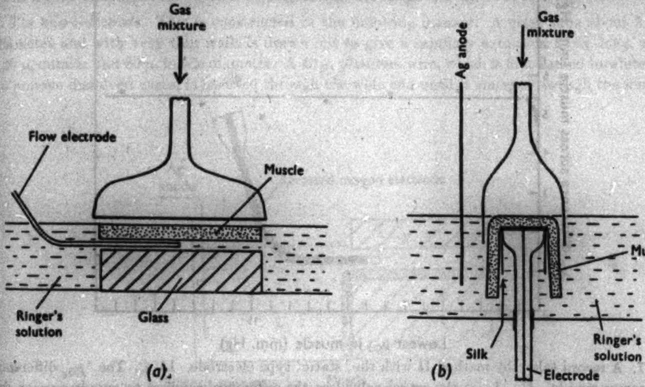

Fig. 6. The arrangement as used in method II. *Not to scale.* In (*a*) the flow electrode is used. The capillary (80 μ. external diameter) lies on the lower glass plate (2 cm. square) and the muscle (250 μ. thick) is stretched over it. The Ringer's solution in the bath is on a level with the upper surface of the muscle. In (*b*) the 'static' type electrode is used. The flat surface of the electrode has a diameter of 12 mm., with the 50 μ. platinum wire flush with the surface at the centre. A layer of silk 150 μ. thick lies on the electrode and is covered by the muscle.

Control of p_{O_2} in the gas mixture. The gas stream consists of cylinder oxygen and nitrogen mixed in variable proportions. Capillary tubes lead from two pressure reservoirs to a mixing T-piece, the bores and lengths of the capillaries being chosen so that a gas mixture of the required proportions can be provided by suitably adjusting the pressures in the two reservoirs. The cylinder nitrogen was found to have a p_{O_2} of not more than 0·1 mm. Hg.

Procedure

The muscle is prepared as in method I. Weights are attached to hold it securely down over the electrode. A gas mixture is applied to the upper surface of the muscle, and after allowing about 4 min. for equilibration V_3 is measured. The proportion of oxygen in the gas is then changed and another reading taken, and so on. The muscle is then removed and the same series of gas mixtures is applied again to the electrode direct, giving V_2. The order of events can be reversed or repeated as desired. The gas mixtures are chosen to include values of V_3 in the region close to zero. The electrode is standardized with air-saturated solution.

Results

The results from one experiment are shown in Fig. 7. This graph is representative of a number that have been made at temperatures varying between 15 and 25° C. There is a certain amount of scatter in the points and the critical p_{O_2} cannot be placed with accuracy to the nearest 0·1 mm. Hg, but, in every case, the first deviation can definitely be said to lie between 0·5 and 2 mm. Hg. As seen in Fig. 7 the deviation is not very great, even below 0·5 mm. Hg, and does not amount to more than about 10% of the p_{O_2} difference at 0·1 mm. Hg.

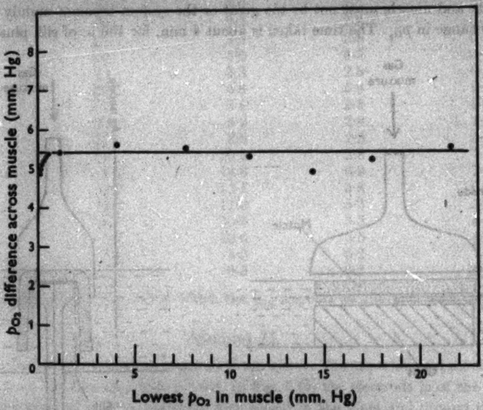

Fig. 7. A record taken by method II with the 'static' type electrode. 15° C. The 'p_{O_2} difference across muscle', $V_2 - V_3$, is the actual value less the difference required to supply oxygen for the electrode reaction, and is proportional to the rate of oxygen consumption of the muscle. The 'lowest p_{O_2} in muscle', V_3, is the p_{O_2} as measured by the electrode.

It should be pointed out that V_3 is a surprisingly good index of the p_{O_2} in the muscle as a whole. It might have been imagined that with a p_{O_2} difference across the muscle of about 6 mm. Hg the greater part of the muscle would be well above the value V_3, with the result that a change in respiration rate at some critical p_{O_2} would not easily be seen on the records. This is not actually the case. Calculation shows that for a muscle of thickness 300 μ., with a total p_{O_2} difference between the two sides of 6 mm. Hg, half the muscle is working at a p_{O_2} less than 1·6 mm. Hg above the minimum, and as much as one-fifth is less than 0·3 mm. Hg above the minimum. Suppose, for example, that when the p_{O_2} fell below 0·3 mm. Hg the muscle ceased to respire altogether: in that case when V_3 approached zero the muscle would behave as though it were 240 μ. thick and the difference across the muscle would fall from 6·0 to 3·9 mm. Hg. The use of V_3 as an index of the 'critical' p_{O_2} is a much more sensitive procedure than might have been imagined.

There is a possibility that the 'critical' p_{O_2} is really higher than one might conclude from these results. If the portion of the muscle that is below the 'critical' p_{O_2} incurs an oxygen debt to make up for its deficient Q_{O_2} and this debt, in the form of a diffusible metabolite such as lactic acid, in being paid off raises the Q_{O_2} of the muscle as a whole to its normal level, the existence of a 'critical' p_{O_2} may be masked. Debt metabolites should, if possible, be removed completely as soon as they are formed, but with isolated muscle it is hard to see how this could be done.

Method III

A brief description of this method has been given above.

The micro-electrode. This is constructed in the following manner. A glass tube about 3 mm. diameter and with very thin walls is drawn out to give a capillary extension 2 cm. long, about 120 μ. outside and 80 μ. inside diameter. A 40 μ. platinum wire, which is first flamed to white heat to remove dissolved gases, is inserted through the wide end until it emerges through the fine end of the tube. The glass at the tip is then sealed around the platinum with a micro-flame, the resulting external diameter being 100 μ. (Fig. 8). The electrode is immersed in boiling aqua regia (4 parts HCl : 1 part HNO_3) and the platinum recessed to a depth of 120–150 μ. This takes about 3 hr. boiling. Finally, with the recess full of water, the electrode is dipped in a solution of 10% collodion in amyl acetate. The collodion film is allowed to dry in air for 3 min., and the electrode is then immersed in water for the collodion to harden.

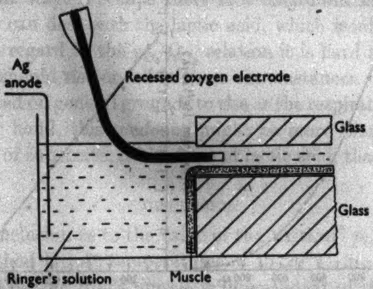

Fig. 8. The arrangement as used for method III with the recessed electrode. *Not to scale.* The space between the muscle and the upper glass plate is 300 μ. deep. The outside diameter of the tip of the electrode is 100 μ., and the depth of the recess 120–150 μ.

This method of making the recessed type of electrode differs from that of Davies & Brink (1942), who did not use aqua regia for the recessing process. This type of electrode, due to its recess, is quite devoid of movement artefact. The collodion film prevents 'ageing' of the electrode—a slow progressive decrease in the electrode current which takes place over a period of hours when the electrode is in contact with living tissue (personal communication from P. W. Davies). The response time of the electrode is about 15 sec. to 90% completion. The current is about 10^{-8} amp. in air-saturated solution.

The electrode is mounted on the rack of a microscope and can be moved horizontally by the fine adjustment screw, the position being adjusted to the nearest 10 μ. The electrode is moved in the fluid space above a flat sheet of muscle which is contained between plates of glass (Fig. 8). Readings are commenced about 3 hr. after setting the upper glass plate into position, with the system then in a steady state. By moving the electrode on the microscope rack, so that its tip travels in a direction at right angles to the edge of the upper glass plate, the p_{O_2} in the still aerobic zone near the edge of the plate can be recorded. The electrode can be moved to and fro as often as required, and the observations thus repeated. The electrode was moved in steps of 125 μ. between readings. The spatial resolution of the electrode is not affected by working in a medium in which there are considerable p_{O_2} gradients. So long as the system is in a steady state the p_{O_2} recorded is that in the fluid exactly at the tip of the electrode.

Provided the rate of oxygen consumption is independent of p_{O_2}, the relation between p_{O_2} and the distance from the boundary is given by the parabola

$$V = V_0 + bx^2/2k - x\sqrt{(2bV_0/k)} \quad \text{(Hill, 1928)},$$

where V_0 is the tension at the boundary, b is the rate of oxygen consumption (allowance being made for the layer of fluid above the muscle), and k is the diffusion coefficient.

At the critical p_{O_2} there will be a deviation from the parabola. To find this point of deviation the derivative of V with respect to x, dV/dx, is calculated and this is plotted against x. The relation is linear so long as the respiration is independent of p_{O_2}, but should cease to be so at the critical p_{O_2}.

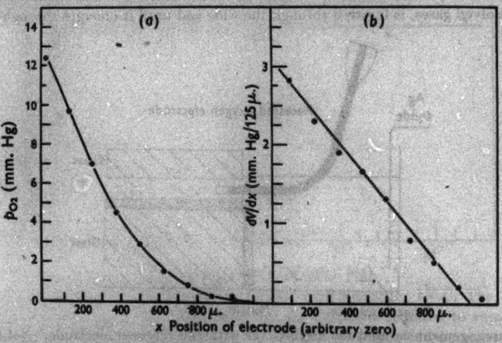

Fig. 9. A record taken by method III. 23·7° C. (*a*) Shows the change of p_{O_2} as the electrode is moved in steps of 125 μ. Each dot represents the mean of six readings. The curve has the form of a parabola. (*b*) The slope of the curve dV/dx is plotted against x. (For a parabola this should be a straight line.) The slope is given as the change in p_{O_2} per 125 μ.

Results

Fig. 9 shows an example. It is noted that the rate of change of p_{O_2} with x becomes quite small as zero is approached, and although the whole aerobic region of muscle (for air outside) is not more than 3 mm. long, the last 2·5 mm. Hg tension covers a distance of about 0·5 mm. The conditions at the lowest tensions can therefore be analysed with considerable accuracy. In Fig. 9 (*b*) dV/dx is plotted against x. The points lie on a straight line to a value of p_{O_2} which is definitely below 0·2 mm. Hg. Other experiments have given similar results, and in each case the critical p_{O_2} is *below* 0·2–0·3 mm. Hg; on the basis of the present observations its precise value cannot be stated. It may be around 0·1 mm. Hg, or it may be lower still; more experiments would be needed to establish it.

These findings are not incompatible with the conclusions arrived at from method II, where it was shown that the respiration started to fall off at a p_{O_2} between 0·5 and 2 mm. Hg, for the conditions are not the same. In method III lactic acid produced in the anaerobic part of the muscle is diffusing into the aerobic zone. It shows up as a continuous slow fall in the p_{O_2} after a time—perhaps 2 hr.—when the steady state in regard to oxygen alone should have been reached. At 3 hr. this change is practically complete, but the respiration of the muscle is by then considerably raised. It is calculated from the observations shown in Fig. 9, which were made 3 hr. after the start, that the rate of oxygen consumption was 2·3 cu.mm./g./min., which is about 5–6 times greater than the normal resting value. Under these conditions it is perhaps remarkable that the relation in the aerobic zone should be so close to a parabolic form as it in fact is. The concentration of lactic acid will decrease towards the outside, and it might have been expected that the rate of excess respiration would fall off in some similar way. Perhaps there is a maximum saturation value at which the muscle can deal with the lactic acid, which would account for this phenomenon. In regard to the p_{O_2}-Q_{O_2} relation it is hard to predict whether the 'critical' p_{O_2} should rise or fall in these circumstances. On the one hand, it might be expected on general grounds to rise as the respiration rate increased, but, on the other hand, this tendency might be more than counteracted by a greater amount of substrate, in the form of lactic acid, than in other parts of the aerobic zone.

DISCUSSION

In venturing a conclusion as to the value of the 'critical' p_{O_2} it is best to offer the results of method II. At temperatures of 15–20° C. it appears, then, that the respiration of resting frog's muscle is independent of the oxygen tension above 0·5–2·0 mm. Hg, and falls off below this value. Other pertinent questions remain to be answered. The effects of temperature changes and of stimulation are important, particularly the latter. In regard to temperature there is the analogy to the behaviour of yeast, mentioned earlier in this paper. Winzler (1941) has shown that the 'critical' tension for yeast is 0·6 mm. Hg at 5° C. and 6·0 mm. Hg at 34° C., a ten-fold change. The metabolic system in muscle is in most ways quite similar to that of yeast and it may be predicted that the 'critical' p_{O_2} in muscle would change to the same kind of extent when the temperature was varied. But what happens when the muscle is stimulated? In a state of maximal activity the rate of oxygen uptake rises about 40-fold over the resting value, and if this extra oxygen goes through the same channels as the resting respiration the 'critical' p_{O_2} would be expected to rise very considerably in activity. In a resting muscle the 'critical' p_{O_2}, around 1 mm. Hg, is so low that the physiological consequences are of little apparent interest. But supposing maximal activity were associated with, say, a 20-fold rise in the 'critical' p_{O_2}, the consequences of this would no longer be insignificant: a large proportion of muscular tissue either in the whole animal or in the isolated muscle would then be respiring at tensions below the 'critical' value, even though no part of the muscle lacked oxygen altogether.

But there is evidence that the respiration at rest is *qualitatively* different from the increased respiration which follows activity. The respiratory enzyme system involved is probably not identical in the two cases, for the extra oxygen uptake during activity is inhibited by sodium azide, while resting respiration is unaffected by the drug (Stannard, 1939). This being so, there is no reason to suppose that the 'critical' p_{O_2} in the active muscle would be higher than it is in resting muscle; it might, in fact, be lower for the *extra* oxygen uptake. In experimenting with active muscle the problem lies in maintaining a constant level of activity over a fairly long period. Drugs such as caffeine will bring the activity respiration system into play up to its maximum capacity, but the rate does not remain constant for long and continuously diminishes with time. In such circumstances an investigation of the p_{O_2}-Q_{O_2} relation would be rather more difficult than it is for resting muscle and remains to be done.

There is a criticism of the technique which has not been resolved. It is this: that so long as there is any possibility of the accumulation of 'debt metabolites' in the muscle, the 'critical' p_{O_2} may really be higher than it would appear to be. If the portion of the muscle that is below the 'critical' p_{O_2} incurs an oxygen debt to make up for its deficient Q_{O_2} and this debt, in being paid off within the muscle, raises the Q_{O_2} towards its original value, the true 'critical' p_{O_2} may be considerably higher than would be inferred from the results given here. Debt metabolites should, if possible, be removed completely as soon as they are formed, just as they would be if the circulation were intact: but it is hard to see how this could be achieved in isolated muscle. Perhaps a much thinner muscle bathed continuously in Ringer's solution would be sufficiently cleansed of 'debt metabolites', or some other technique might be developed. So far the criticism has not been properly answered.

SUMMARY

1. A study is made of the influence of oxygen tension on the rate of respiration of resting isolated frog's muscle. The oxygen tension is measured by means of the oxygen electrode.

2. The respiration is constant and independent of oxygen tension above 0·5–2·0 mm. Hg partial pressure, at temperatures of 15–20° C. The rate falls off as the tension is further reduced.

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AFFERENT NERVE FIBRES FROM THE HEART AND LUNGS IN THE CERVICAL VAGUS

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During the course of experiments on the behaviour of stretch receptors in the lung, a number of fibres in the cervical vagus nerve were encountered in which the impulses were grouped in time with the heart beat. Some were ordinary stretch fibres with occasional groups of impulses showing a cardiac rhythm, as has been described by Adrian (1933), and some were aortic depressor fibres. In addition, there were some whose pattern of activity was unfamiliar, and a systematic search was therefore made in the cervical vagus for fibres giving cardiac rhythms. Attempts were made to identify the natural stimulus to each type of fibre by recording venous and arterial pressures in the systemic circulation during various respiratory manœuvres. The existence of two kinds of afferent cardiovascular fibres in the vagus has already been briefly reported (Walsh & Whitteridge, 1944, Walsh, 1947). Recently, a paper by Amann & Schaefer (1943) has become available in which impulses from fibres arising in veins are unmistakably recorded.

METHODS

Satisfactory results have been obtained from twenty-one cats and from a single rabbit. In the earlier experiments cats were decerebrated and given small doses of chloralose (10 mg./kg.) to diminish reflex excitability. In such preparations the vagi were usually white and bloodless, and no activity could be found, apart from that due to stretch fibres from the lung. The number of unsuccessful experiments was reduced but not abolished by using intact cats deeply anaesthetized with chloralose (80 mg./kg.) after induction with ethyl chloride. The vagus was dissected between the nodose ganglion and a cervical branch of the carotid artery, and care was taken to preserve the nutrient vessels to the trunk. In these animals the trunk bled freely when its sheath was incised, and fibres with cardiac rhythms could usually be found. The right vagus was usually used, but no differences between the two sides have been observed. Another advantage of this approach high in the neck was that the depressor nerve often arises in the cat from the superior laryngeal nerve near to the vagus trunk, and could easily be identified. The sheath of the vagus was split with a von Graefe cataract knife and removed gently. A nerve bundle was cut at the central end, separated for about 1 cm. and laid on the electrodes. If any cardiac rhythm was audible in the loud-speaker, the bundle was further subdivided. A binocular loupe made this stage a little less uncertain. The action potentials were amplified by a conventional resistance capacity coupled amplifier and recorded with a double beam cathode-ray tube and camera. The second beam was used for recording the e.c.g. usually from lead III. For immediate recognition of the timing of volleys, the amplified *QRS* wave was used to trigger the beginning of the sweep circuit, so that it was possible to distinguish visually between a presystolic and postsystolic outburst. With heart rates of 180 per min., there are no other methods of identifying the time relations of a volley.

Simultaneously, the pressures in the arch of the aorta, the superior vena cava and the pleural cavity were recorded by mirror membrane-manometers of the Hamilton-Wiggers type. The transmission in the first two was by heparin saline in lead tubing, in the third by air in pressure tubing. A lumbar puncture needle was passed down the left carotid artery to within 1–2 cm. of the aorta, and a gum elastic catheter was passed about 10 cm. down the external jugular vein.

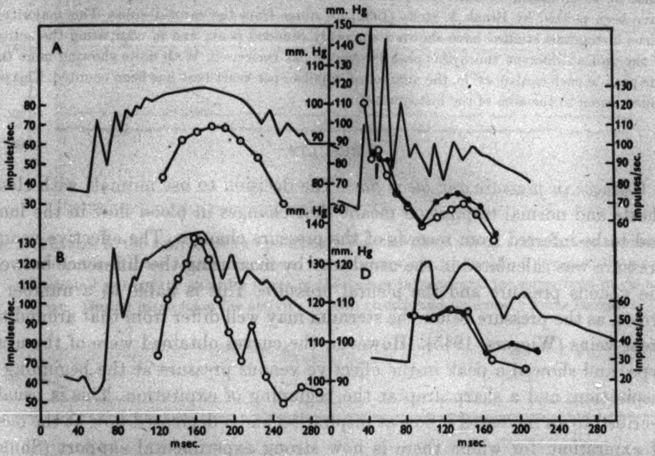

Fig. 1. The relation between the pressure wave recorded near the arch of the aorta and the activity in single units in the depressor nerve. The reciprocal of the interval between successive impulses is plotted as the frequency, in impulses per sec. Time is measured from the *Q* wave of the e.c.g., which was recorded simultaneously. In C and D, two units could be identified in the record from the depressor nerve. In C, the cat was decerebrate, in the remainder it was anaesthetized with chloralose. Pressure wave, continuous line. Nerve impulses, —o—.

A wide bore needle with multiple apertures was passed into the right pleural cavity, so that its point lay below the sternum, and about 2 ml. of air were injected. The natural period of the arterial manometer varied from 100 to 200 cyc./sec., and that of the venous manometer was barely high enough at 50–90 cyc./sec. The animal's head and chest were in a shielded warm moist chamber. For cooling, two copper and silver foil troughs were constructed on the pattern used by Partridge (1939), and cooled by cold brine.

In the later part of this work some technical modifications introduced by Torrance (Torrance & Whitteridge, 1947) made the separation of the fibres somewhat easier. The cooling chambers were fed with alcohol cooled by circulation through freezing mixtures, and a number of methods of using the compression technique of Leksell (1945) were tried. Compression of the nerve by sponge rubber for 10–15 min. and by an inflatable membrane system were both very effective. More single fibres were obtained in the course of one afternoon using the compression method than had been seen in 3 months of isolation of single fibres by manual dissection.

A set of action potentials, if uniform in amplitude and regularly spaced, has been assumed to be due to activity in a single unit. For convenience, time intervals have been measured from the *Q* or *R* wave of the electrocardiogram recorded simultaneously. The reciprocals of the time intervals between impulses have been plotted against the mid-points of the time intervals. The resulting histograms from aortic depressor fibres have been plotted alongside the optical records of blood-pressure pulses (Fig. 1). The position of the arterial cannula was checked at the end of the experiment. The imperfections in the optical record, worst in Fig. 1C, are due to too low a natural period of the manometer system. The records have been corrected for errors due to misalignment, but not for the conduction time of the nerve impulses. This should not exceed 3–5 msec. It can be seen that, though the histogram of the nerve impulses may lead or lag relative to the pressure pulse by 10–20 msec., its contour is related to the contour of the pressure pulse. Similar curves have been plotted by Bronk & Stella (1932) for fibres from the carotid sinus. The majority of nerve histograms studied have shown a smoothly rounded peak, and in comparing the activity of the unit at different times this peak frequency has been used. With units showing more than one peak in each cardiac cycle, the number of impulses per heart beat has been counted. This is of course equal to the area of the histogram.

RESULTS

Changes in pressure and blood flow. The decision to use animals with closed chests and normal respiration meant that changes in blood flow in the lungs had to be inferred from records of the pressure changes. The effective venous pressure was calculated in the usual way by measuring the difference between the venous pressure and the pleural pressure. This is liable to a number of errors, as the pressure below the sternum may well differ from that around the great veins (Wiggers, 1945). However, the curves obtained were of the usual type, and showed a peak in the effective venous pressure at the beginning of inspiration, and a sharp drop at the beginning of expiration. This is usually ascribed to an increased inflow at inspiration and a decreased flow at the onset of expiration, for which there is now strong experimental support (Shuler, Ensor, Gunning, Moss & Johnson, 1942; Dupee & Johnson, 1943).

The right ventricular pressure has been recorded by plunging a needle through the chest wall into the right side of the heart, but this has not been satisfactory. Good records have been obtained for short periods, but, as it is impossible to prevent the needle from moving during respiration, no reliance can be placed on the direction of the pressure changes.

At the beginning of inspiration there was usually a fall in the systemic arterial pressure, but occasionally there was no change. These changes in effective venous pressure and arterial pressure were accentuated during the inspiratory efforts which follow obstruction of the trachea during expiration. They can be safely ascribed to retention of blood in the lungs during the period of rapid expansion, and an increased outflow of blood into the left auricle at expiration (Cahoon, Michael & Johnson, 1941; Dupee & Johnson, 1943).

During artificial inflation of the lungs with positive pressures applied to the trachea, the intrapleural pressure rises to a smaller extent. The effective venous pressure in the great veins may rise or fall, the right-ventricular pressure rises, and the aortic pressure falls (Fig. 5). When the lungs are allowed to collapse, the effective venous pressure rises sharply, especially in decerebrate animals in which the muscular tone is high. The aortic pressure continues to fall for

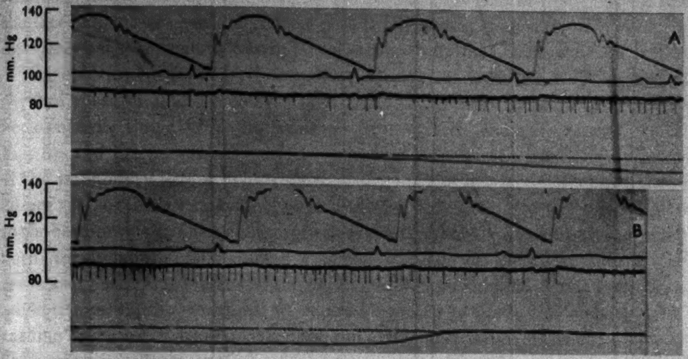

Fig. 2. Changes in the activity of two pulmonary vascular fibres during a single normal breath. Records A and B are continuous. From above downwards, aortic blood pressure; the e.c.g.; stretch and pulmonary vascular fibres in the vagus; intra-pleural pressure; time in $\frac{1}{10}$ and $\frac{1}{100}$ sec. During inspiration the activity of both stretch and pulmonary fibres increases.

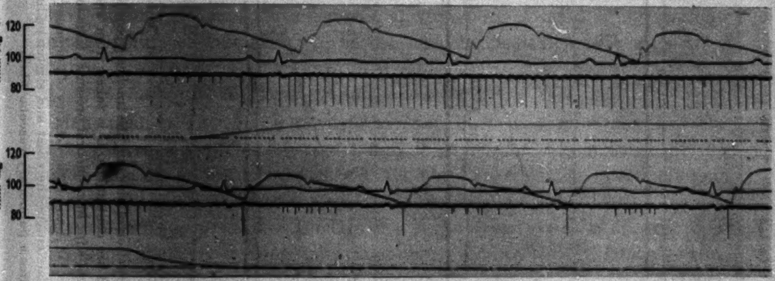

Fig. 3. The effect of positive pressure inflation of the lungs on pulmonary vascular fibres. From above downwards, aortic pressure; the e.c.g.; stretch and pulmonary vascular fibres in the vagus; intra-pleural pressure; time in $\frac{1}{10}$ and $\frac{1}{100}$ sec. The period of inflation is signalled by the rise in the intra-pleural pressure, and is accompanied by discharge from a stretch ending and by abolition of activity in the pulmonary vascular fibres.

a beat or two, and rises slowly, not reaching its peak until after the second breath following deflation. This is to be attributed to the emptying of the lungs of blood by the positive pressure (Kuno, 1917). When the positive pressure is removed, and inflow of blood from the great veins is permitted, there is

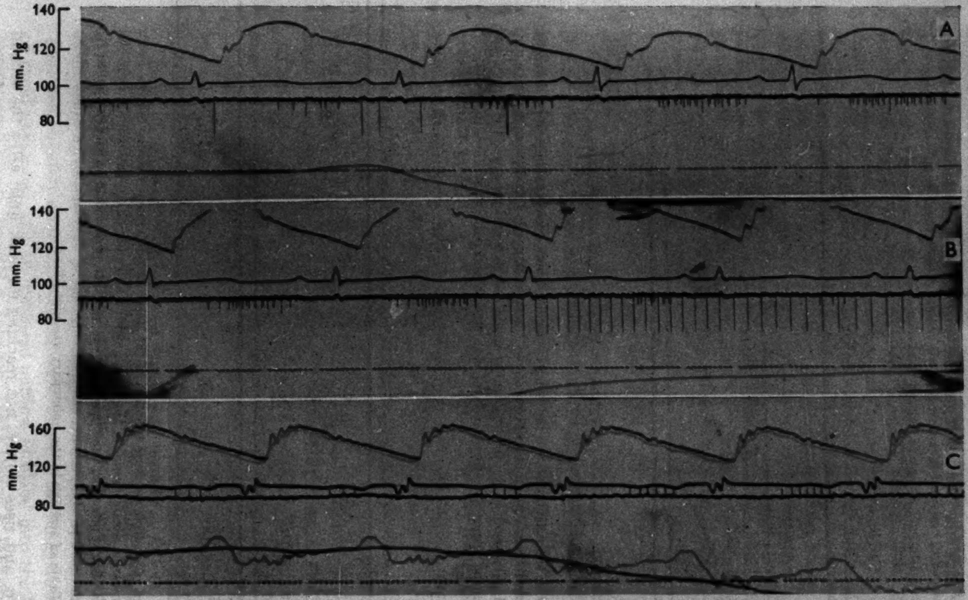

Fig. 4. The effect of suction of air from the trachea (records A and B), and attempted inspiration with the trachea closed (record C). Records A and B are continuous, C is taken from another experiment. From above downwards, aortic pressure; the e.c.g.; impulses in a vagal slip; intra-pleural pressure; time in $\frac{1}{10}$ and $\frac{1}{100}$ sec. In addition, in C only, a record of the pressure in the superior vena cava. In A and B the activity of pulmonary vascular fibres is greatly increased during suction, and is diminished at its release, when air enters the chest and stretch endings discharge. In C an attempted inspiration lowers the intra-pleural pressure and excites a pulmonary vascular fibre.

a 'wave-front' of blood which travels slowly through the pulmonary circuit.

In artificial deflation of the chest by suction of air from the trachea the effective venous pressure rises and the arterial pressure usually falls at first (Fig. 4), but the later effects are variable. Holt (1944) has shown that suction frequently interferes with the venous return by collapsing structures at the thoracic inlets.

The electrical activity of nerve fibres. Groups of impulses with a cardiac rhythm are often found in nerve fibres in the vagus (Fig. 2). Such volleys have been seen in single fibre preparations from stretch endings (Adrian, 1933; Fig. 3), and are presumably due to pulsation transmitted from the branches of the pulmonary artery. The volleys are usually of low frequency, not above 50 per sec., occurring in systole or in diastole, with a slightly variable relation to the cardiac cycle. Their characteristic feature is that the discharge is increased both during normal and during artificial inflation of the chest, and as the respiratory stimulus increases, the cardiac rhythm decreases. Apart from these adventitious cardiac rhythms, there are a number of fibres which show 'true' cardiac rhythms. Normal inspiration and artificial inflation of the chest usually have opposite effects on these fibres (Figs. 2, 3). They show short volleys of high frequency, exactly related to the cardiac cycle, and as a rule their amplitude is lower and the duration of their impulses is longer than in those of the stretch fibres. No confusion with the rapidly adapting stretch fibres reported by Knowlton & Larrabee (1946) is likely, as the vascular endings are active during the expiratory pause and with normal rates of inspiration. Further points of difference will be discussed in connexion with each type of fibre.

Systemic arterial pressure receptors

Many records of activity in the depressor nerve have been published (Rijlant, 1932; Adrian, 1933), but there are few, if any, records from single fibres. Subdivision of the trunk is not easy as the fibres are fragile and probably few are normally inactive. When the peak frequency of discharge is plotted against the systolic pressure there is sometimes a slight departure from linearity due to concurrent changes in intrapleural pressure. Sometimes the discharge can be stopped completely by pulling on the carotid cannula. The aortic endings lie in the adventitia (Nonidez, 1937), and appear to be affected to some extent by mechanical changes at the root of the lung. A few fibres have been seen which run in the carotid sheath separate from the depressor or vagal trunks, having probably arisen from the innominate-subclavian region. On cooling, depressor fibres are blocked at 10–12° C., at a little lower temperature than the stretch fibres. This is true for the whole trunk, as well as for preparations containing only a few fibres. When a depressor fibre has had a pronounced respiratory rhythm, it has been related to an unusually large respiratory oscillation in blood pressure, with its peak at the end of expiration.

In four experiments, groups of impulses have been seen very early in systole. There is little doubt that these impulses must arise during the isometric contraction phase, before the opening of the aortic valves (Fig. 8). Some evidence for the presence of depressor fibres which arise from the left ventricle was put forward by Daly & Verney (1927), but the impulses observed here have the further peculiarity that they may increase in activity during inflation of the chest by positive pressure, at a time when the systemic arterial pressure was observed to fall. They are, therefore, unlikely to arise from the left ventricle. One fibre was observed during an extra systole which appeared in the e.c.g. and the venous pressure record, but from the record of aortic pressure evidently failed to open the aortic valves. There was, however, an outburst of impulses in this fibre. In view of the results of Dawes (1947), on the injection of veratrine into the coronary artery, the possibility that these fibres may arise from the right ventricle or the septal region needs further investigation. The observations of Johnson (1947), on the pressures in the ventricular wall itself, suggest that endings in the thickness of the wall might be the source of these impulses.

Fibres from the veins

The pulse waves from the great veins have been recorded simultaneously with the action potentials and e.c.g. (Fig. 9). As is generally accepted, these pressure records show an *a* wave roughly midway between *P* and the *QRS* complex, an early small *c* wave just after the *QRS*, a depression during the emptying of the ventricle, and a *v* wave reaching its peak after the *T* wave and the dicrotic notch, when the auriculo-ventricular valves open. With heart rates of about 180 per min., the diastolic pause is reduced, and the *v* wave may run into the next *a* wave. The *v* wave is somewhat variable in height, and may be almost absent, when the heart rate is high (Wiggers, 1945, Fig. 3).

The *c* wave is probably not an artefact. Its peak coincides with the opening of the aortic valves. In some experiments the *c* wave is inconspicuous.

In three experiments, the effective venous-pressure curves have been obtained by subtraction and compared with the pattern of impulses in the venous fibres. There has always been general agreement, but plotting of pressure against frequency of discharge gives a wider scatter than, for example, occurs with aortic fibres. Occasionally a *v* volley of impulses begins before the *v* pressure wave can be seen (Fig. 10). There is, however, correspondence between *a*, *c* and *v* waves and *a*, *c* and *v* volleys of impulses over a cardiac cycle, over a respiratory cycle, and after increasing the venous return by abdominal compression. It is, therefore, probable that these fibres arise from endings situated on the great veins or the right atrium.

In experiments with the chest open and positive pressure ventilation, only depressor and venous fibres have so far been detected in the vagus. In these conditions, venous fibres are almost uninfluenced by the respiratory changes, and in one experiment they were not affected by obstruction of the pulmonary veins sufficient to raise the pulmonary arterial pressure by 10 mm. Hg. In this last experiment it seems safe to conclude that the venous fibres arose from the right and not from the left side of the heart. With an intact chest, activity in venous fibres is increased in normal and obstructed inspiration (Fig. 9), and may be increased or decreased during positive pressure inflation. When *v* volleys are present, they show greater changes with respiration than do the *a* volleys. In general, the behaviour of these fibres can be predicted from a knowledge of the changes in the effective venous pressure.

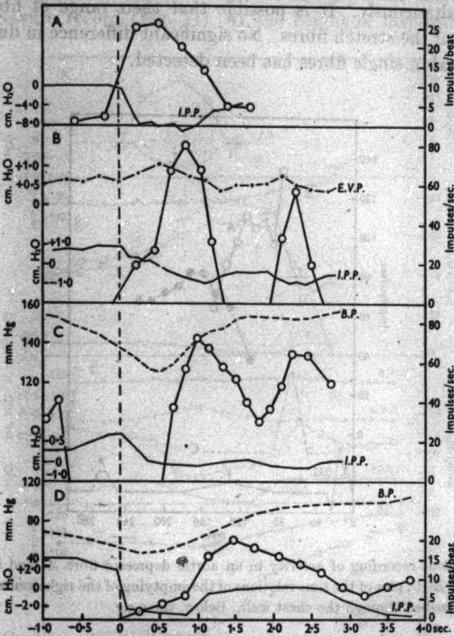

Fig. 5. The effect of the release of a period of positive pressure inflation of the chest on A, a fibre apparently signalling venous pressure from the great veins; B and C, pulmonary vascular fibres; and D, a fibre apparently signalling pressure in the great veins. In all cases, the positive pressure is released at time 0. *I.P.P.* intra-pleural pressure; *E.V.P.* effective venous pressure; *B.P.* aortic blood pressure; —o—, activity of nerve fibres, recorded as the number of impulses per heart beat in A and D, and as the peak frequency of impulses in each heart beat in B and C.

On cooling, fibres from the veins are blocked by a temperature of 8–12° C. Stretch fibres usually drop out at slightly higher temperatures, 12–16° C., but the separation obtained in this way is not sharp. Venous fibres are resistant to the earlier stages of anoxaemia. The amplitude of their action potentials is usually smaller, but has sometimes been greater than that of stretch fibres recorded simultaneously. It is possible that their range of fibre diameters overlaps that of the stretch fibres. No significant difference in duration of the action potential in single fibres has been detected.

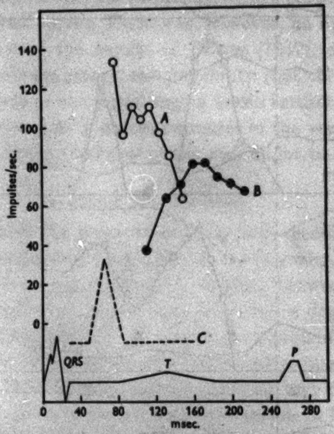

Fig. 6. Simultaneous recording of activity in an aortic depressor fibre *A*, and in a pulmonary vascular fibre *B*. *C*, plot of the time relations of the emptying of the right ventricle, as recorded by a needle pushed through the chest wall. Below, the e.c.g.

Two fibres in separate experiments have been observed at some length because they showed *a*, *c* and *v* volleys, but their activity changed quite independently of the effective venous pressure (Figs. 5, 7). Their activity decreased at the beginning of normal inspiration, and increased sharply at the onset of expiration. When the flow of blood through the lungs was checked by positive pressure inflation, they stopped discharging, and only began again at the end of the first inspiration following collapse. The usual venous fibres, on the other hand, show a sharp rise in activity the moment the pressure in the lungs is released (Fig. 5). It is suggested that these two exceptional venous fibres arose from endings in the pulmonary veins or left atrium, where appropriate endings exist (Nonidez, 1941). Increased filling of the left atrium at expiration has been observed by Cahoon *et al.* (1941).

The pulmonary vascular receptors

The third and probably the commonest type of cardiovascular fibre was characterized by a late systolic volley, with a very pronounced increase in activity during inspiration. The systolic volley appeared later than the discharge in the depressor fibres (Fig. 6), and usually later than the peak in the aortic pressure record (Fig. 11). At the height of inspiration there was occasionally an impulse or two in time with the *c* wave of the atrial pulse. The majority of fibres show a rounded peak in their frequency-time curve at about 200 msec. after the *Q* of the *QRS*, but a few fibres, whose behaviour is otherwise indistinguishable, have shown an initial peak reminiscent of the arterial pressure pulse (Fig. 11 A).

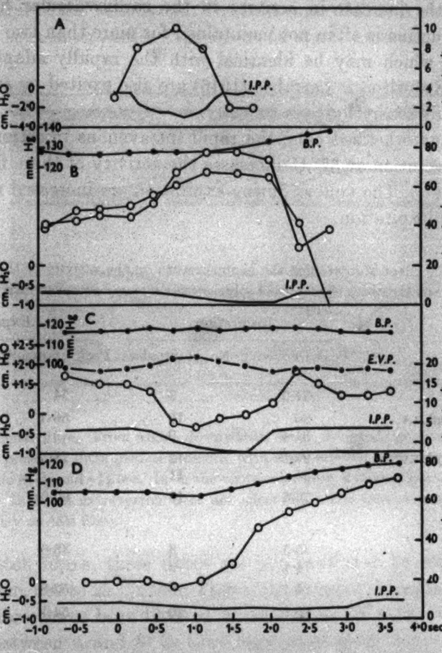

Fig. 7. The effect of a normal inspiration on A, a right venous fibre; B, two pulmonary vascular fibres; C, a left venous fibre; and D, an aortic depressor fibre. *I.P.P.*, intrapleural pressure; *B.P.*, aortic blood pressure; *E.V.P.*, effective venous pressure.

During normal inspiration the number of impulses in each volley, and the peak frequency attained, increase three- or four-fold. One fibre became a little less active in normal inspiration, but it was strongly stimulated, like all other such fibres, during the inspiratory efforts which follow closing of the trachea in expiration (Fig. 4). During this time there is little or no change in the activity of the stretch fibres, since no air enters the chest. Suction of air from the trachea provides a less satisfactory way of distinguishing between these fibres and stretch fibres (Fig. 4). This is because stretch fibres are frequently stimulated by collapse as well as by expansion of the lung (Adrian, 1933), and also because the increase in activity in the cardiovascular fibres which is produced by suction is often not maintained for more than two or three heart beats. Fibres which may be identical with the rapidly adapting receptors described by Knowlton & Larrabee (1946) are also excited by suction, but do not show a cardiac rhythm.

In deeply anaesthetized cats, the rapid intravenous injection of 20 ml. of a 0·9% NaCl solution at 37° C. increases the activity of these fibres for some minutes (Table 1). The volleys during expiration are increased more than the volleys during inspiration.

TABLE 1. The effect of increasing the venous return on the activity of the pulmonary vascular fibres (0·9% NaCl solution was injected intravenously)

	Inspiration		Expiration	
	Peak frequency	No. of impulses	Peak frequency	No. of impulses
Exp. 1:				
At rest	71·2	9	54	2
After 20 ml. I.V.	80	10	56·3	6
	81·4	9		
After 40 ml. I.V.	97·1	12	78·5	10
	85	11		
20 min. later	76·6	13	60·6	9
Exp. 2:				
At rest	75·5	8	38·6	3
	74·4	9		
After 20 ml. I.V.	85·1	8	53·8	4
3 min. later	80	10	54·1	4

During artificial inflation of the chest, the activity of these fibres is reduced or abolished, although the stretch fibres continue to signal the volume of air in the chest, independent of the intra-tracheal pressure (Fig. 3). When the chest is allowed to empty, there is a sharp rise in the venous pressure a beat or two later (Fig. 5). After one or two further heart beats, this type of cardiovascular fibre shows a great increase in activity, which precedes by some seconds the return of the aortic pressure to its initial value. This behaviour makes it unlikely that these fibres arise in any system directly dependent on the aortic pressure, such as the coronary or bronchial circulation, but is to be expected if their endings lie in the pulmonary circulation.

Obstruction of the trachea at the height of inspiration is followed by active expiratory efforts which make the pleural pressure more positive and abolish activity in the pulmonary fibres. In general, their activity varies with the magnitude of the negative pleural pressure, and with the blood flow through the lungs. As a working hypothesis it is suggested that they signal the distending pressure in the arterioles or capillaries of the lung. Possibly fibre A in Fig. 11 lies close to the arteriolar end of the capillary, while the more usual type B lies nearer to the venous end. The behaviour of fibre A closely resembled that of a fibre seen in an earlier experiment on starch embolism (Walsh, 1947).

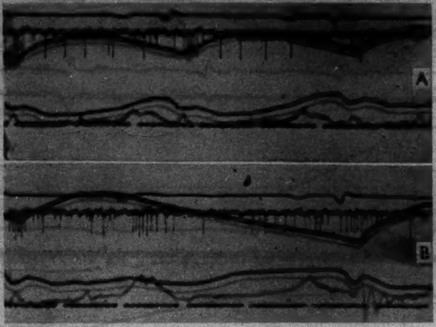

Fig. 8. A ventricular fibre. From above downwards, e.c.g., inverted; impulses in a vagal slip; aortic blood pressure; intra-pleural pressure, with superimposed cardiac waves; right venous pressure, time in $\frac{1}{10}$ and $\frac{1}{100}$ sec. In A, the ventricular fibre, a stretch fibre and a venous fibre are present. In B, an extrasystole does not affect the aortic pressure curve, but does give rise to a volley on this fibre.

Unlike stretch fibres, these fibres are not paralysed by exposure to 3% trichlorethylene, nor to 25% ether. They remain active after stretch, depressor, and venous fibres have been blocked by cooling to 10° C. and only disappear at 3–4° C. Between 4 and 8° C. they may show some degree of Wedensky inhibition. If cooled to just below 3° C. the block is readily reversible on warming, but no recovery has been seen in nerves which have been frozen. Quick freezing and thawing, which was found by Head (1889) to separate inflation and deflation reflexes in the rabbit, was of no value in the cat.

In order to measure the duration of these impulses the *QRS* complex was locked to the beginning of the sweep of the oscillograph in the usual way. A stimulus could be taken from this sweep at any point on its course and used to trigger a second sweep travelling across another oscillograph at a much higher speed. In this way about 10 msec. of any part of the cardiac cycle could be examined in detail. By setting the stimulus appropriately it was possible to measure the duration of impulses in early systole from the aortic fibres, in late systole from the pulmonary vascular fibres, and in presystole from the venous fibres. Impulses from venous and arterial fibres were found not to differ significantly in duration from those from stretch fibres. Pulmonary vascular fibres gave an impulse duration of 0·058 ± 0·03 (10) msec. compared with stretch fibres at 0·36 ± 0·02 (11) msec. This difference is highly significant.

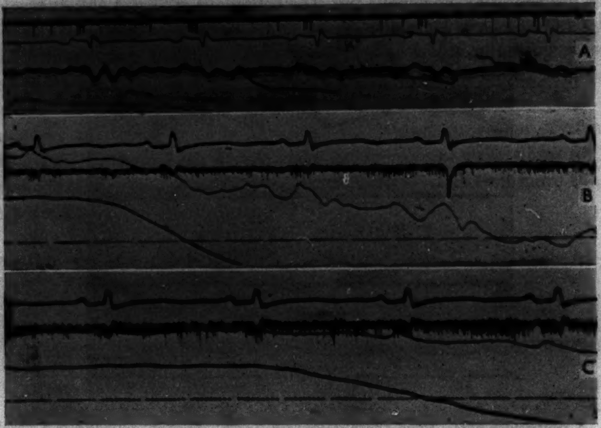

Fig. 9. Fibres from the veins. A, from above downwards, a venous fibre with a stretch fibre coming in just before the last heart beat; the e.c.g., inverted; a venous pressure record; arterial blood pressure, which is rising throughout the record; B and C are taken from the same experiment. There are fewer venous fibres and a more sensitive venous manometer in B. From above downwards, the e.c.g.; the venous impulses; the venous pressure record; the intrapleural pressure; time in $\frac{1}{10}$ and $\frac{1}{100}$ sec.

Occasionally, there are no signs of these fibres in the bundles excised from the vagus. It has been usual to ascribe these failures to bloodlessness of the nerve, or slight cyanosis of the entire animal, but attempts to obtain evidence of the sensitivity of these fibres to anoxia, by administering nitrogen while leading from them, have been inconclusive. From their durations these impulses must run in γ or δ fibres, and it has been shown by Grundfest (1939) that δ fibres are second only to B fibres in their sensitivity to oxygen lack. Attempts to measure their conduction rate have been unsuccessful.

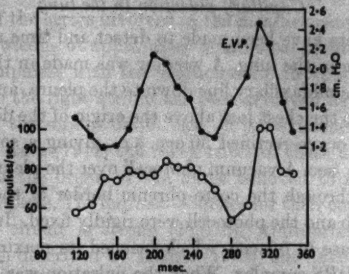

Fig. 10. The relation between the effective venous pressure in the superior vena cava, —●—, and the activity in a single venous fibre, —○—. The first elevation in effective venous pressure is the *v* wave, and the second is the *a* wave of the next heart beat. Time in msec. measured from the *R* wave of the e.c.g.

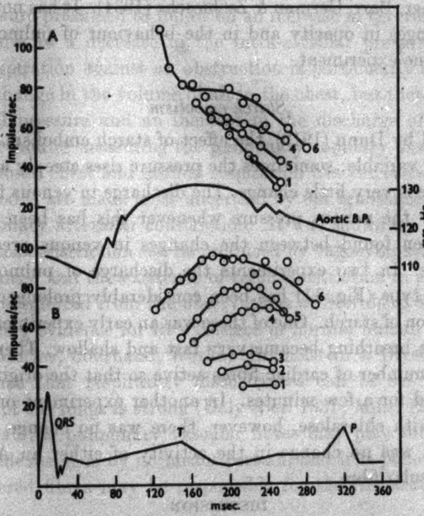

Fig. 11. Superimposed records of the activity in two pulmonary vascular fibres (A and B) during successive stages of inspiration, 1–5, and during suction of air from the trachea, 6. A single representative aortic blood-pressure curve and an e.c.g. are also shown, though minor changes in duration of the cardiac cycle and in the amplitude of the pressure pulse occurred with respiration.

Capillary pulsation in the lung

A few experiments have been made to detect and time any capillary pulsation there might be in the lung. A window was made in the chest wall in the seventh space in the midaxillary line down to the pleura, and a small light bulb was introduced into the chest just above the origin of the diaphragm. The bulb was heated by full-wave rectified 50 cyc. a.c., giving a source of light which flickered at 100 cyc./sec. A vacuum photocell over the pleural window received light transmitted through the costo-phrenic border of the lower lobe of the lung. Both the bulb and the photocell were rigidly fixed. In some experiments there was an increase in opacity which reached its maximum 150–180 msec. after the *Q* of the *QRS* complex. When the pulsation was not present at rest, it was produced by the injection of 100 μg. adrenaline. Its peak was at 180 msec. after the *Q* wave, that is about 40 msec. later than the peak of the arterial pressure pulse. It has been shown by Lewis (1924) that capillary pulsation normally occurs in the finger when the blood flow is rapid, and in the intact lung in situ capillary pulsation has been observed on occasion by Wearn, Ernstene, Bromer, Barr, German & Zschiesche (1934). It has not been possible to observe changes in opacity and in the behaviour of pulmonary vascular fibres in the same experiment.

Starch embolism

As was found by Dunn (1919), the effect of starch embolism on the venous pressure is very variable; sometimes the pressure rises steeply and stays high, sometimes there is very little change. The discharge in venous fibres has been found to follow the venous pressure whenever this has been recorded. No relation has been found between the changes in venous pressure and the respiratory rate. In two experiments the discharge of pulmonary vascular fibres of the A type (Fig. 11) has been considerably prolonged into diastole after the injection of starch. One of these was an early experiment on a decerebrate cat whose breathing became very fast and shallow. There was a great increase in the number of cardiac fibres active so that the original fibre could only be followed for a few minutes. In another experiment on a cat deeply anaesthetized with chloralose, however, there was no change in respiratory rate, and there was no change in the activity of either an A or a B type pulmonary vascular fibre.

DISCUSSION

There are comparatively few experiments available in which venous pressures and satisfactory records of single units from endings presumed to lie on the great veins have been studied simultaneously. This is partly due to the fact that these fibres are particularly difficult to isolate, and many records have been found to contain more than one unit. However, the agreement between venous pressure and the numbers of impulses in these fibres is as good as can be expected in view of the errors involved in the measurement of effective venous pressure. Our knowledge of appropriate anatomical endings is complete, and the recording of impulses with the characteristic rhythm in branches of the vagus which run to the right atrium has been achieved by Amann & Schaefer (1943). In view of the serious doubt cast on the existence of the Bainbridge reflex by Ballin & Katz (1942) the reflex effect of these fibres is uncertain.

The evidence for the origin of the fibres believed to arise from the lung vessels is also incomplete. The indirect evidence for their connexion with events in the pulmonary circuit is strong (Fig. 5). Appropriate endings on lung arterioles have been described by Takino (1933), but have not been confirmed by Elftmann (1943). Further work on this point is being undertaken. These fibres could be responsible for the cardiovascular reflexes which arise in the lung (Schwiegk, 1935; Daly, Ludany, Todd & Verney, 1937).

If these endings lie on the walls of the alveolar capillaries, they should be stimulated by the distending pressure in these vessels. They should, therefore, increase their rate of discharge in response to an increase in the pulmonary capillary pressure presumed to follow on an increase in the venous return, and also in response to a decrease in the intra-alveolar pressure. The effect of attempted inspiration against an obstruction is particularly striking as there is almost no change in the volume of air in the chest, but there is a sharp drop in the pleural pressure and an increase in the discharge of the pulmonary fibres. No method of measuring the pulmonary capillary pressure under conditions of a closed chest and normal respiration has yet been devised, and all indirect estimates are made most uncertain by our ignorance of the extent of normal pulmonary arteriolar constriction. It was shown by Daly & v. Euler (1932) that such constriction can be produced by vagosympathetic stimulation in perfused lungs, and the existence of tonic constriction in some decerebrate animals can be inferred from the observations of Sternberg & Tamari (1928). Recently, strong evidence for its existence in normal human beings has been provided by Motley, Cournand, Werko, Himmelstein & Dresdale (1947).

The evidence that respiratory disturbances can be set up by vascular engorgement of the lungs is strong (Daly *et al.* 1937; Mills, 1944). Although it is possible that the pulmonary vascular fibres may play some part in these respiratory reflexes, it is by no means certain, and it is possible that some as yet undiscovered fibres may be responsible for the 'inspiratory drive' from the lungs.

SUMMARY

1. A number of nerve fibres in the cervical vagus show activity corresponding to the cardiac rhythm. This can be distinguished from that due to aortic depressor fibres, and from adventitious cardiac rhythms in pulmonary stretch fibres.

2. Evidence is put forward to support the view that some of these fibres arise from endings on the roots of the great veins or on the adjacent right atrium. Occasionally, fibres may be found which probably arise from the pulmonary veins or left atrium.

3. A considerable number of fibres have been found which are believed to arise from endings on the small vessels of the lung. Their impulses have a longer duration than those in stretch fibres, and survive cooling to 4–8° C.

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OSTEOGENESIS AROUND MULTIPLE FRAGMENTS OF CHICK EMBRYO BONE GRAFTED TO DEVELOPING CHICK EMBRYO CHORIOALLANTOIS

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A previous paper reported the survival and rapid vascularization of single fragments of chick embryo bone grafted to developing chick embryo chorioallantois (Hancox, 1947).

In the present experiments, multiple fragments grouped in close proximity were employed. New bone was laid down between and around the grafts.

METHODS

The method employed was as before, with minor modifications. Hosts and graft-fragments were again from embryos of 10 and 14 days' incubation respectively, and the living preparations were available for direct microscopical observation. Multiple fragments, generally three, were used as grafts. They were grouped in close proximity in the plasma clot on the cover-slip. Exposure of the host chorioallantois was made toward the lower pole of the egg, further from the airspace than formerly; sagging away of the chorioallantois during the early stages of incubation was thereby almost eliminated.

The preparations were photographed daily, considerable improvement in the technique having been effected. A high-pressure Hg vapour lamp was used as light source, its rays being condensed to fall obliquely upon the specimen, which was maintained vertically in a warm box mounted on the moving stage of a horizontal microscope. For visual purposes, the ultra-violet band was filtered off; for photographic purposes the unfiltered radiation was employed and seemed innocuous. Non-red sensitive emulsion was employed on the photographic plates, giving satisfactory contrast between blood vessels and bone.

At the end of the experiments the bone-bearing areas of the host membranes were excised. The modified von Kossa silver method for bone salt calcium 'staining', described by McLean & Bloom (1940) and discussed by Bloom & Bloom (1940), was employed. Gold toning was carried out on some preparations. No sections were cut, the finished preparations being cleared and mounted in toto.

Observations were concentrated mainly upon the living preparations and their photographic records; the impregnated in toto specimens were used to confirm the bony nature of newly formed material.

RESULTS

General course. As before, the bone fragments soon became vascularized; usually all three grafts of each preparation were vascularized simultaneously, but sometimes one or more fragments lagged by 24–48 hr.

During the first 2 or 3 days, the vascular pattern of each fragment remained entirely separate; later, however, increasing anastomosis appeared between the vessels of different fragments, and it was usual to find a single large efferent vessel whose tributaries took origin in all three fragments (Pl. 2, fig. 7). The impression was thus gained, teleologically speaking, that the fragments, treated by the host as initially discrete, later became components of a local unified system.

As the days elapsed the vascular pattern showed great augmentation, fresh vessels constantly appearing both around and within the bone. Fresh bone usually first made its appearance on the third day; it was considerable by the fourth day and increased steadily over the succeeding days.

The experiments were generally terminated by the sixth day when quite large bone deposits had formed; thereafter, there was a tendency for the membrane to become loosened from the cover-slip and to pulsate vigorously, precluding photographic record.

Osteogenesis. The new bone was formed both between fragments and also in the form of spicules, extending outwards from the grafts.

Bone between fragments was occasionally seen on the second day; it usually appeared on the third and was unmistakable by the fourth day (Pl. 1, fig. 2; Pl. 2, fig. 8). It consisted of trabeculae, of varying thickness, which tended to bridge the gap between adjacent fragments, though not necessarily by the shortest route. The trabeculae often seemed to take origin from both fragments and soon became broader and stouter.

Peripheral bone spicules varied greatly in size; at one end of the scale there were quite short and blunt outgrowths hardly extending beyond the original cut edge of the fragment. This new bone could be seen to be laid down in continuity with the trabeculae of the graft fragments which may therefore be said to have grown. At the other end of the scale relatively long and delicate spicules extending out over the surface of the host membrane, as shown in Pl. 2, figs. 8, 9, were also seen.

Relationship of developing bone to the vascular system

The close relationship between the vascular and the trabecular pattern is demonstrated in Pl. 1, figs. 4, 5. A mass of bone formed by the complete fusion of three fragments is shown; surface remodelling has led to the deposition of radially arranged trabeculae, running parallel to capillaries which drain into a central efferent vessel.

New bone was constantly observed to arise in areas previously well vascularized. For example, in Pl. 2, fig. 6, the two top fragments of bone are connected together by vessels which can be discerned with some difficulty (but which were obvious in the original preparation); 48 hr. later (Pl. 2, fig. 7) extensive bone deposition has taken place at the same site.

DISCUSSION

It seems uncertain whether or not, during its development, the trabecular pattern of bone is determined by its vascular system or vice versa. Gebhardt (1905) and Weidenreich (1923) consider that alterations in bony architecture, the result of mechanical stress, are brought about by changes in the vascular supply; on the other hand, Jores (1920) and Petersen (1930) are of the opinion that changes in the vascular supply are secondary to changes in the bone itself. Murray (1936) discusses the point fully. Lately (Murray, 1947), evidence has been obtained from birefringence studies that the microscopic architecture of bone is related rather to its vascular pattern than to external form or mechanical function (Melon & Dallemagne, 1943; Dallemagne & Melon, 1943). That development of a trabecular pattern may occur quite independently of the vascular system, is of course, shown by the occasional occurrence of osteogenesis in portions of detached knee-joint cartilage persisting as 'loose bodies' in man, and also by tissue culture experiments (Glucksmann, 1939). Weiss (1934) was able to control the direction of nerve fibres growing in tissue cultures by mechanical modification of the ultra-structure of the substratum, and it seems possible that the orientation of the fibre scaffold of the bone developing in the present experiments might also be laid down in such a fashion as to follow lines or orientated protein micelles. Such micellar orientation might be brought about by local mechanical stresses, such as movement initiated by the developing embryo, or even by the presence or growth of capillaries.

The present experiments support the view that, at least under certain circumstances, the histoarchitecture of bone tissue is a function of its vascular supply. They do not confirm the views of Leriche & Policard (1928), who state that 'the newly formed spicules of bone are always situated as far as possible from the blood capillaries which enter the non-ossified connective tissue spaces. In their immediate neighbourhood where stagnation is least there is never bone formation.'

The occurrence of osteogenesis uniting the fragments requires some consideration. It has been shown (Fell, 1932) that cells derived from the endosteum of long bones from the chick embryo are osteogenic in vitro. The cells growing out from the fragments in the present experiments are, at least in part, of similar derivation; and it can safely be assumed that the new bone arose in association with them in a manner similar to the tissue culture experiments. Further work is in progress with the object of ascertaining why bone should form beyond the confines of the grafts when multiple but not when single fragments are employed.

The rather rapid appearance of new bone in association with the multiple fragments affords, perhaps, additional evidence for the view that clinical chip-grafts may survive the grafting operation and serve as a highly osteogenic nidus.

SUMMARY

1. Multiple juxtaposed fragments of chick embryo bone, on grafting to developing chick chorioallantois, survive and become vascularized.

2. Osteogenesis occurs, so that the fragments tend to become united. Peripheral spicules also form.

3. In a broad sense the trabecular pattern was determined by the vascular pattern, bone formation occurring in relation to vessels.

Thanks are due to R. McV. Weston of Simpl, Ltd., for advice regarding photographic technique.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Egg 120. Appearance at 24 hr. Two of the three fragments are partly obscured by haemorrhage; vessels were present in all three, but do not show clearly. The outlines of the fragments are distinct. (× 60.)

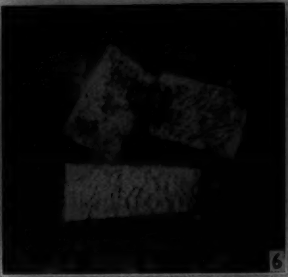
Fig. 2. Same, 3 days later. Note almost complete bony union between fragments, accomplished by relatively thick, wide trabeculae especially in the central zone, between fragments. (× 60.)

Fig. 3. Portion of same, 48 hr. later. Complete bony union of two fragments can be seen. A haemorrhage is present in one place. Note vessels within bone. (× 115.)

Fig. 4. Egg 114. 5 days' incubation. Three fragments have fused. A large efferent vessel, obscured by haemorrhage, was present toward the centre of the mass. (× 60.)

Fig. 5. Portion of preceding. Surface remodelling has been accomplished by laying down of trabeculae in association with capillaries draining radially into the efferent vessel. (× 115.)

Figs. 1–5.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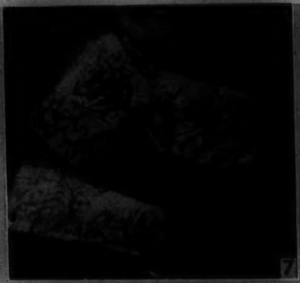
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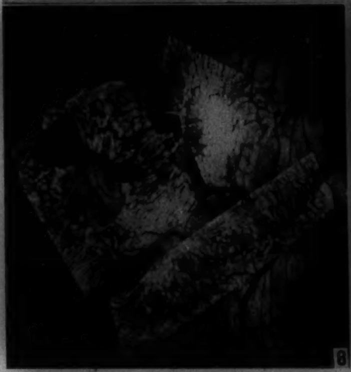
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Figs. 6–9.

PLATE 2

Fig. 6. Egg 96. 48 hr. incubation. Vascular interconnexions were present between the two upper fragments, but do not show clearly. (× 60.)

Fig. 7. Same, 48 hr. later. Bone has formed in region indicated in previous figure. Note also a large efferent vessel into which blood drains from all three fragments. (× 60.)

Fig. 8. Egg 135. 4 days' incubation. Thin interfragment trabeculae have formed, originating from both of the two upper fragments. A tenuous spicule protrudes upwards from the upper surface of the left-hand upper fragment; there is a haemorrhage near its point of origin. (× 60.)

Fig. 9. Same, 24 hr. later. The trabeculae have widened and thickened; the spicule is now considerably longer. An artificial defect was created in the left-hand fragment prior to the start of the experiment. (× 60.)

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THE PRESSURE DEVELOPED IN MUSCLE DURING CONTRACTION

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There is much speculation, but little is known for certain, about the connexion between chemical breakdown in muscle and the physical events of contraction and relaxation. It is not even certain that in the elementary unit of muscular activity, the single twitch, contraction and relaxation are associated with any of the chemical processes as yet recognized. It is true that during the course of a maintained contraction chemical changes can be shown to occur, particularly the breakdown of creatine phosphate and (if the stimulus is prolonged) the formation of lactic acid: but a maintained contraction is the resultant of many elementary contractions and relaxations, fused more or less together to outward appearance, and overlaps the early stages of recovery from all except the last of these, so that any chemical changes observed at the end may be connected rather with recovery. It is assumed by some that the breakdown of adenosine triphosphate (ATP) provides the energy for contraction, or, by others, for relaxation: but since no changes at all have been found in the ATP of living muscle, except in extreme fatigue verging on rigor, it is not possible to say when (or if) its breakdown actually occurs in a normal twitch.

The initial heat production in the twitch of a frog's muscle, being 2 to 3×10^{-3} cal./g., corresponds, if the breakdown of ATP or phosphagen be supposed to account for it, to about 2×10^{-7} mole of phosphate, or about 6 μg. of P, set free per g. of muscle. This assumes Meyerhof's (1947) figures of 11,000 cal./mole of phosphate from phosphagen, or of 12,000 cal. from ATP. Contraction and relaxation occupy about 1 sec. at 0° C., and about 0·2 sec. at 20° C. The supposed chemical changes, therefore, are so small and so rapid that direct chemical methods cannot possibly cope with them. Very special value, therefore, is attached to any other method, with sufficient sharpness of resolution in time and sufficient sensitivity and specificity, which might be used to establish a connexion between contraction and relaxation on the one hand and chemical changes on the other. One such method is that of measuring the heat production. This is sensitive and accurate, and can be made very rapid, but it does not distinguish specifically between one chemical change and another. The heat production in a twitch occurs in the contractile phase only: in relaxation no heat at all appears unless mechanical energy is degraded into heat. The measurement of pH is more specific, but as hitherto employed it is affected by the lag of the electrode (Dubuisson, 1939, p. 465) and by the time taken in diffusion from the inside of the muscle: together these must require several seconds before a true reading is obtained. The measurement of volume changes is another method which is sensitive and rapid, though not specific: indeed it provides the only evidence yet available (apart from the heat production) that chemical changes have actually occurred in a single twitch by the time that relaxation is complete. Unfortunately, its application is affected by a serious complication which it is the purpose of this paper to describe.

The complication is this. A considerable mechanical pressure is developed inside a muscle when it contracts, which is bound to cause a compression, followed in relaxation by a decompression, of its contents, particularly its contained water. The early and reversible changes of volume accompanying contraction and relaxation must be largely, if not entirely, due to this. The later observed effects, the remainder at the end of relaxation and any subsequent changes, are unaffected by this compression and may safely be attributed to chemical processes. The early volume changes, however, must be so largely affected by it that they cannot be used directly as an index simply of chemical breakdown, and the hoped for resolution in the early stages is not realized.

The first measurement of volume change during contraction was made by Ernst (1925). Versfelt (1927) confirmed its existence and attributed it to mechanical compression of the contents of the muscle: indeed he estimated the pressure developed during contraction of a frog's gastrocnemius as about 100 mm. Hg. The most significant results, however, were obtained by Meyerhof and his colleagues and are referred to below.

Water is a rather compressible substance, its volume change at 15° C. being about $4{\cdot}9 \times 10^{-5}$ c.c./c.c. × atm., as compared with mercury $0{\cdot}37 \times 10^{-5}$, copper $0{\cdot}05 \times 10^{-5}$, and iron $0{\cdot}04 \times 10^{-5}$. In the papers of Meyerhof & Möhle (1933), Hartmann (1934) and Meyerhof & Möhle (1935) are a number of records of the volume changes of a frog's gastrocnemius undergoing an isometric tetanus. There is a rapid diminution of volume immediately after the stimulus begins, the volume continues to diminish as the stimulus goes on, and when the stimulus ends there is a sudden increase of volume coinciding in time with relaxation. The net result, when relaxation is complete, is a diminution of volume which can safely be attributed to chemical breakdown, probably chiefly of creatine phosphate. The reversible effect seen in contraction and relaxation can most readily be assessed by measuring the sudden increase in volume during relaxation. The rapid reversible decrease of volume at the beginning of contraction is complicated by the possibility of rapid onset of the decrease associated with chemical breakdown: they cannot easily be separated. During relaxation, however, the volume returns rapidly to a nearly constant base-line.

The following results (Table 1) were measured off from all the figures in the three papers for which a calibration is given: in each case the contraction was the first of a series, providing usually the greatest reversible effect.

TABLE 1. Increase of volume in relaxation

Author	Tetanus (sec.)	Volume increase (c.c./g. muscle)	Note
Meyerhof & Möhle (1933)	4	$0{\cdot}8 \times 10^{-5}$	
	2	$1{\cdot}5 \times 10^{-5}$	
	4	$2{\cdot}6 \times 10^{-5}$	Muscle previously fatigued
	0·2	$7{\cdot}4 \times 10^{-5}$	*
	2·0	$7{\cdot}5 \times 10^{-5}$	*
Hartmann (1934)	3	$0{\cdot}7 \times 10^{-5}$	
	2	$1{\cdot}3 \times 10^{-5}$	I.A.A.
	2	$0{\cdot}9 \times 10^{-5}$	I.A.A.
	2	$0{\cdot}6 \times 10^{-5}$	
Meyerhof & Möhle (1935)	0·8	$1{\cdot}9 \times 10^{-5}$	

Of these results, two, marked by an asterisk, are much larger than the rest. They are given on the same page (478), and were obtained presumably under similar conditions; probably there is some abnormality about them and they have been omitted from the mean. The next largest reading was taken under similar conditions on a muscle which had been subjected to a previous series of stimuli. Two records (I.A.A.) were on muscles poisoned with iodoacetic acid. A mean value for all, except the two marked with asterisks, is $1{\cdot}3 \times 10^{-5}$ c.c./g. of muscle. If we assume that the compressibility of muscle is the same as that of water, this would require a mean pressure of about 200 mm. Hg to produce it.

In single isometric twitches a similar measurement of four records in papers by Meyerhof & Möhle (1935) and Meyerhof & Hartmann (1934) gave for the volume increase in relaxation $1{\cdot}35 \times 10^{-5}$, $3{\cdot}7 \times 10^{-5}$, $1{\cdot}8 \times 10^{-5}$ and $0{\cdot}9 \times 10^{-5}$ c.c./g. The scatter is too wide to allow a fair average to be taken. The mean given by Meyerhof & Hartmann is 2×10^{-5}, but from this must be subtracted the remainder after relaxation, leaving (say) $1{\cdot}5 \times 10^{-5}$. The reversible volume changes for an isometric twitch are obviously about the same as for a tetanus.

In the isometric twitch of a frog's gastrocnemius, the rise of temperature (Hill, 1931) is not more than 3 to 4×10^{-3}° C. If this could be attributed to the dephosphorylation of ATP or of creatine phosphate it would require (at 12,000 cal./mole of phosphate split off) about 3×10^{-7} mole of one or the other to be broken down: this (at 10 c.c. volume constriction per mole—Meyerhof 1947, p. 824) would give a diminution of volume of about 3×10^{-6} c.c./g. of muscle, which is only about one-seventh of the average $(2{\cdot}0 \times 10^{-5})$ observed by Meyerhof & Hartmann. Clearly, known chemical changes are inadequate to account for the volume constriction observed in a twitch.

Can an average pressure of 200 mm. Hg be developed inside a frog's gastrocnemius during an isometric contraction? Direct evidence that it can is given below, but strong indirect evidence exists already in the well-known cessation of blood flow in a strongly contracting muscle. Barcroft & Millen (1939), for example, found that the flow of blood in a human gastrocnemius was completely stopped by an isometric contraction which was not more than one-third maximal. If the arterial pressure (say 130 mm. Hg) was overcome by the pressure set up inside the muscle in such a sub-maximal contraction, a maximal one should have been capable of producing a pressure of about 400 mm. In a dog's gastrocnemius Anrep & Saalfeld (1935, fig. 2) showed an immediate cessation of arterial flow during a tetanus, though the contraction was a weak one (only 2·7 kg.). How are these pressures produced? The structure of the gastrocnemius is very complex, with fibres only half the length of the muscle as a whole (Hill, 1931) pulling in various directions. The external form of the gastrocnemius shows that the fibres and their tendons cannot be lying straight, and if they are curved they must necessarily exert a pressure inwards when they contract, depending on their tension and radius of curvature. The smaller size of the frog's gastrocnemius does not imply a smaller pressure: with a similar form and the same intrinsic strength of contraction the greater curvature would exactly compensate for the smaller number of fibres. We should expect, therefore, in a frog's gastrocnemius, a pressure to be developed, during an isometric contraction, of the order of several hundred mm. of Hg.

All the recorded experiments on volume change during contraction were made on frogs' gastrocnemius muscles, except those of Fischer (1941) on a frog's sartorius. Fischer's results are not described in detail, and the changes found varied in size, and even in direction, with initial length: it is difficult to draw any conclusion from them.

In order to settle the matter it was necessary to measure this pressure, if it existed, directly. The method employed depends on a novel principle, the rise of temperature associated with the adiabatic compression of oil. Like air, any fluid with a positive coefficient of thermal expansion will show a rise of temperature when compressed. One should choose a liquid with as large a thermal coefficient as possible. Various fluids exist (e.g. pentane) with a coefficient of about $1{\cdot}6\times10^{-3}$ c.c./c.c. × ° C., but these are all unsuitable to be injected into a muscle. Thin medicinal paraffin oil, however, is very suitable and it has a fairly high coefficient (about $7{\cdot}6\times10^{-4}$; compare water at 17° C., $1{\cdot}5\times10^{-4}$). The rise of temperature on compression can be calculated from the formula

$$\delta T=\frac{\alpha T\delta P\times10^{6}}{(\text{density})\ (\text{specific heat})\ 4{\cdot}18\times10^{7}},$$

where α is the coefficient of thermal expansion, T is the absolute temperature (taken as 290° K.), δP is the pressure in megabars (which can nearly enough be taken as atmospheres) and $4{\cdot}18 \times 10^7$ is the mechanical equivalent of heat (see e.g. Poynting & Thomson, 1904). Inserting the constants for paraffin oil the rise of temperature per atmosphere works out at about 0·012° C.

METHODS

A fine hypodermic needle was blocked at its tip and a small hole was ground near the tip on one side. It was fitted to a small syringe, the piston of which was modified to carry a 'thermistor' mounted on fine manganin wires above it. The piston was movable, but could be fixed by the turn of a screw. The syringe and needle were filled completely with the paraffin oil referred to above, no air bubbles being allowable since these would buffer the oil against change of pressure. The syringe was mounted on a screw stand by which its needle could be driven forward accurately and conveniently into a gastrocnemius muscle held isometrically on a board. When the needle had been inserted, usually at an angle of about 25° with the axis of the muscle, the hole near its tip being well inside, about 0·01 c.c. of oil was forced in by pressing on the piston, which was then fixed. This oil was intended to spread around the needle and to ensure that its small hole was not blocked by tissue lying across it: it was necessary that any pressure developed should be communicated at once to the oil in the needle and piston. When the muscle contracted, the pressure inside it rose; this raised the pressure in the syringe (the flow in the needle must have been very small), the temperature of the oil rose and lowered the resistance of the thermistor, and a galvanometer (see below) deflected. When the muscle relaxed, the pressure fell, the oil cooled and the galvanometer returned.

The thermistor (type V 597, kindly given me by Dr E. J. Baldes of the Mayo Foundation, Rochester, Minnesota) is a glass-coated spheroid of about 0·3 mm. diameter with two 25 μ. platinum alloy leads. It contains a semi-conducting material with a very high-temperature coefficient of resistance, about 3·3% per 1° C. The specimen used had a resistance of about 2000 Ω at 17° C. Being so small it takes up the temperature of the oil very quickly, in less time than is needed to read the galvanometer.

The arrangement was calibrated directly. By means of a pump, an air-pressure reservoir, a two-way tap and a manometer, the pressure inside a thick rubber tube containing oil could be suddenly raised or lowered by a known amount above or to atmospheric. The needle was forced through the rubber into the oil, so that known changes of pressure could be suddenly produced in the syringe. The pressure was turned on (or off) and the deflexion of the galvanometer was read; the result was expressed in terms of change of resistance in the thermistor. A rise of pressure of 1 atm. lowered the resistance by 0·74 Ω. A resistance was kept in series with the thermistor, which could be suddenly switched in or out, so allowing the galvanometer deflexion to be expressed in terms of pressure change. As finally used, the galvanometer gave 1 mm. deflexion for a pressure change of 10 mm. Hg.

For accurate work, the syringe would have to be maintained at a constant temperature and the thermistor connected to a resistance bridge. Since no great accuracy was desired but only to find out whether changes of pressure do occur in a stimulated muscle, and of what order of size, a simpler arrangement was used. The thermistor was placed in series with 33,000 Ω and a battery (24 V.), and the potential difference between the ends of the thermistor was led, through a good 8 μF. condenser, to an amplifier. The output of the amplifier was led through a similar condenser to the galvanometer (period 1 sec.). Slow changes of temperature, therefore, caused no deflexion of the galvanometer, which responded only to rapid changes. Calibration was carried out under the same conditions as the experiments on the muscle, so the deflexions were comparable.

The current in the thermistor (about 0·7 mA.) produced enough heat to warm the oil slightly in its neighbourhood. Movement of the oil, therefore, if allowed, would cool the thermistor, but pressure changes caused so little movement that no error of this kind occurred: a fall of pressure gave the same deflexion, but in the opposite sense, as an equal rise of pressure: movement of the oil would have caused a cooling of the thermistor in either case. The deflexions obtained with muscles were always sharply reversible, a rise of temperature (corresponding to a rise of pressure) on contraction, a fall of temperature (corresponding to a fall of pressure) on relaxation.

RESULTS

The results were definite though somewhat variable. Gastrocnemius muscles of English *Rana temporaria* were used. Insertion of the needle into the muscle was bound to cause some injury and successive stimuli (tetani of 1 or 2 sec. duration) gave diminishing deflexions. One could not be sure (i) that the hole in the needle was in the most suitable place inside the muscle, (ii) that some leakage of oil did not occur along the line of the needle when the muscle contracted, or (iii) that the muscle did not draw away slightly from the needle (which was inserted from the lower end) even in a contraction intended to be isometric. All such causes would tend to reduce the rise of pressure recorded, and doubtless combined to give the variability observed.

The observed deflexions corresponded to pressures from 100 to 300 mm. Hg. For example, successive readings on one muscle were (*a*) 300, 200, 160, 200, 100: and with the needle inserted in a new place 170. Other series were (*b*) 250, 170, 170, 150, 150; (*c*) 130, 240, 200, 100, 100, 100; (*d*) 130, 90, 60, 140, 100; 130, 120, 120, 140, 110, 100; 170, 130, 150. No significance would attach to a mean value, particularly since every experimental influence tends to reduce the reading. It is clear, however, that a pressure of the order of 200 mm. Hg is, in fact, developed inside a frog's gastrocnemius during contraction—which is sufficient to account, at least in isometric contractions, for the reversible part of the volume changes observed.

DISCUSSION

Meyerhof and his colleagues examined the volume changes in isotonic as well as in isometric contractions: in the isotonic ones the muscle was completely unloaded before contraction, and then allowed to shorten freely—either an unlimited amount, or until it came up against the tension recording device. The volume changes observed were much smaller in isotonic than in isometric contractions, as regards not only reversible effects but also the remainder after relaxation. The relative smallness of the reversible effects could be expected because the force exerted by the fibres of a muscle allowed to shorten freely would be much reduced and the pressure developed be correspondingly less. The smaller remainder after relaxation is an index of smaller chemical change. The heat production in a muscle initially unloaded and allowed to shorten freely may be very much less than when initially stretched. Even in single twitches, for example, Cattell (1932) found that a gastrocnemius initially unloaded and allowed to shorten freely gave out less than one-quarter of the heat observed in an isometric contraction under a standard initial load: this effect I have observed in a sartorius under similar conditions. In a tetanus the effect may be even greater, since the later stimuli are applied to a muscle already considerably shortened, and the heat may be far less than under isometric conditions or with a considerable isotonic load. A smaller heat production implies a smaller chemical change. These two factors, therefore—smaller mechanical force and smaller chemical breakdown—can amply account for the fact that the volume changes found in unloaded isotonic contractions are much smaller than in isometric.

Whether the much smaller reversible volume changes under isotonic (unloaded) conditions also are due to compression cannot be decided by the present experiments, for the measurement of pressure would have been unreliable had the muscles been able to move considerably. Prof. Meyerhof writes me from Philadelphia: 'The best and most reliable measurements done formerly in Heidelberg are in the 1935 paper (pp. 538, 539). From these I calculate that the volume increase in relaxation (c.c. per g. of muscle) for the isometric twitch is $1{\cdot}3 \times 10^{-5}$, for the isotonic $0{\cdot}5 \times 10^{-5}$. For the tetanus the respective values are $1{\cdot}9 \times 10^{-5}$ and $0{\cdot}5 \times 10^{-5}$.... While I would agree that most of the reversible volume change in isometric contraction is water compression, I would think that most of the relatively small reversible change in isotonic contraction is not.' At any rate, the approximate identity in size of the reversible volume changes in twitch and tetanus shows that they cannot be associated with the chemical processes of which the heat is an index, for the latter is far greater in a tetanus. It is possible that the complex form of the gastrocnemius prevents its fibres from shortening freely as much as they could, in which case a pressure would be developed even when the muscle was unloaded. Without more evidence it is clearly unsafe to use the reversible volume changes as an index of chemical or physical processes, other than mechanical ones, accompanying contraction.

If, therefore, we admit that the rapid reversible changes of volume are due mainly to mechanical compression, we are left with the remainder after relaxation as a sign of chemical breakdown. There is a small remaining constriction after even a single twitch, which—from the form of the curves—appears to have accumulated during contraction, or relaxation, or both. In magnitude it would correspond approximately to the amount of ATP or phosphagen broken down, as calculated (see p. 518) from the heat production. After a tetanus the remaining constriction is larger; it has undoubtedly accumulated during contraction, as is seen from the continuing decrease of volume while the stimulus goes on. After relaxation the constriction usually diminishes—the volume increases again—corresponding probably to the resynthesis of phosphagen and the formation of lactic acid. In a muscle poisoned with iodoacetate, however, the constriction may go on increasing after relaxation (Hartmann, 1934, p. 175), corresponding to a continued breakdown of phosphagen (cf. Lundsgaard, 1934; D. K. Hill, 1940, p. 473; Dubuisson, 1939). If, therefore, accurate allowance could be made for the reversible volume changes due to mechanical compression, we could obtain by subtraction, even during contraction and relaxation, a very significant curve of volume change almost certainly associated with chemical breakdown. The best way to apply this correction would probably be to assume (i) (as above) that, as regards magnitude, the sudden increase of volume during relaxation is due to decompression, and (ii) that, as regards time course, compression and decompression follow the recorded curve of isometric tension. Better still, however, would be to work with muscle containing only straight parallel fibres, in which changes of pressure would not, presumably, occur.

The result would be very interesting. Whether the early constriction (after allowance for the reversible changes) is due to phosphagen breakdown alone, or whether ATP breakdown provides any part of it, cannot be decided on the present evidence, but the continuing decrease of volume of an I.A.A. muscle after relaxation can scarcely be due to the resynthesis of ATP in the Lohmann reaction at the expense of phosphagen breakdown, since the volume changes, molecule for molecule, of these two processes should nearly balance each other (+10 c.c./mole for ATP resynthesis, −11·5 c.c./mole for phosphagen breakdown: Meyerhof, 1947).

If experiments on volume changes in muscle are to be repeated, it is suggested that they ought to be made at 4° C. At this temperature the density of water is a maximum, so that much less accuracy is required in the thermostat and the heat production of the muscle itself would cause no error. A further advantage of the low temperature would be that events in muscle go on much more slowly and do not require so sharp a resolution in the recording apparatus.

SUMMARY

1. A method is described of measuring alterations of pressure by means of the reversible change of temperature caused by the compression of oil.

2. In the maximal isometric contraction of a frog's gastrocnemius an internal pressure is developed of the order of 100–300 mm. Hg.

3. The same pressure should be developed in a muscle of any size, provided it be of the same shape and the same intrinsic strength. It explains the cessation of blood flow during strong contraction.

4. This pressure is sufficient to account for the reversible changes of volume accompanying contraction and relaxation, which can be regarded as due to mechanical compression and decompression, chiefly of the water contained in the muscle.

5. This does not affect the accepted view that the decrease of volume remaining after relaxation, and its subsequent changes, are due to chemical breakdown.

I am grateful to Mr V. H. Attree and Mr A. C. Downing for their help in constructing and setting up the apparatus, and to Dr E. J. Baldes for the gift of the thermistor.

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Acapnia due to decompression. By W. D. M. Paton.

National Institute for Medical Research, Hampstead, London, N.W. 3

From accounts of unaided submarine escape, it is clear that it is easier to refrain from inhaling during ascent through water than might be expected. One contributing factor would be the loss of CO_2 from the lungs by venting air during the ascent. For an ascent at rate S, with lung volume V, and surrounding pressure P, the rate of venting is $(V \times S)/P$, and the amount vented in an ascent from depth D to the surface (by integration) is $V \log_e D$. For $V = 5$ l. and $D = 60$ ft., amount vented $= 5{\cdot}2$ l. If the subject's minute volume before the ascent was about 5 l./min., there should thus be a lengthening of breath-holding time of about 1 min.

To test this, the subject (W.D.M.P.) determined his breath-holding time (in these conditions, the time during which it was possible to refrain from inspiration) at zero pressure, under pressure and during decompression. A mouthpiece connected to two water valves was used, with which it is easy to avoid unintentional inspiration, yet the lungs can vent freely in decompression. Decompressions took about 2 min. and ended shortly before breaking-point. In the last series, at the breaking-point, alveolar air samples were taken to verify that the end-point was constant.

Results are shown in the following table; O_2 and CO_2 tensions are given in percentage of an atmosphere:

Pressure (ft. of water)	Breath-holding time (sec.)	Remarks
0	68·2, 70·4, 79·3	(Breath-holding time determined after a maximum inspiration in all tests)
30	122·8, 123·1	
60	115·6, 127·1	
Decompression 60 to 10 ft.	223	Breath held 90 sec. before decompression started
Decompression 60 to 10 ft.	182	Breath held 60 sec. before decompression started
0	(*a*) 59·1	(*a*) —
	(*b*) 74·7	(*b*) CO_2 7·10%; O_2 11·65%
60	(*a*) 111·5	(*a*) CO_2 7·81%; O_2 49·3%
	(*b*) 111·5	(*b*) CO_2 7·75%; O_2 49·6%
Decompression 60 to 11 ft.	178·2 (breath held 60 sec. first)	CO_2 7·94%; O_2 15·01%
Decompression 60 to 10 ft.	205·2 (breath held 90 sec. first)	CO_2 7·98%; O_2 13·2%

These results show that the breath-holding time under pressure is roughly double that at the surface, and that the breath-holding time with interposed decompression is roughly doubled yet again. This further increase thus cannot be due to the increase in oxygen tension while under pressure, and is also shown not to have involved any extension in the physiological breaking-point. It is of interest that a greater advantage is obtained the longer the breath is held before the decompression; the concentration of CO_2 in the air vented and hence the total loss of CO_2 is thereby increased. Taking this effect into account, the increases in breath-holding time due to decompression (60–100 sec.) are of the order expected, if they were due to a partial acapnia resulting from loss of CO_2 in the air vented from the lungs during decompression.

Delayed onset of rigor mortis after administration of myanesin.

By E. C. Bate-Smith and J. R. Bendall. *Low Temperature Research Station, University of Cambridge*

As a qualitative observation, activity of an animal at or shortly before death has a considerable effect on the rate of onset of rigor mortis in the muscles. Barbiturate (dial) anaesthesia gives inconsistent results, since muscular relaxation is frequently not achieved. The new muscle-relaxing drug myanesin (α:β-dihydroxy-γ-(2-methylphenoxy)-propane, Berger & Bradley, 1947), administered intraperitoneally at the rate of 0·3 g./kg. body weight 30 min. before the animal is killed, produces in rabbits a marked delay in the onset of rigor.

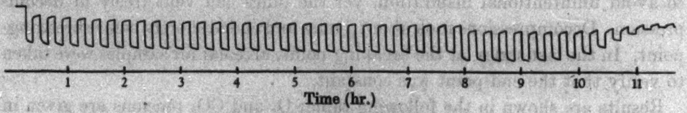

Fig. 1. Myanesin-treated rabbit.

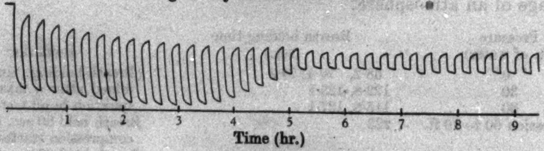

Fig. 2. Normal rabbit.

Figs. 1 and 2 show the effect of myanesin on the course of rigor mortis in the excised psoas muscle, as compared with that of an untreated animal.

Rigor mortis was registered at room temperature as described by Bate-Smith (1939), with the difference that loading and unloading were effected automatically. A complete cycle consisted of 8 min. with the load on (downward stroke), and 8 min. with the load off (upward stroke). The onset of rigor is indicated by a decrease in extension under load. In the myanesin-treated animal there was a delay of 10 hr. before this point was reached, in the untreated animal of only 4 hr. (This duration was, in fact, rather unusually long for a normal animal; the delay, which is subject to great variation, is more usually 1–2 hr. only.) In a second myanesin experiment the delay was 7 hr.

Since the onset of rigor is associated with the breakdown of adenosine triphosphate (ATP) in the muscles (Bate-Smith & Bendall, 1947), it is suggested that myanesin may be useful in the preparation of ATP in enabling an increased yield to be obtained.

This work forms part of the programme of the Food Investigation Board Department of Scientific and Industrial Research Acknowledgement is made to the British Drug Houses Ltd. for the gift of myanesin used in this work.

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Ranine ventricular dimyophysitism. By W. Burridge.

After sufficient perfusion, the frog's ventricle is revealed to consist of two kinds of muscle, the pale and the pink. The right half is pink, the left half is pale. The two kinds of muscle differ in their physiological reactions. Pale muscle is more susceptible to the action of potassium salts than is pink, and adrenaline acts more on pink muscle than pale. Adrenaline, when strong enough, can produce a 'sore' in pink muscle.

Augmentation of output is specially associated with augmentation of effort of pink muscle.

animal there was a delay of 16 hr. before this point was reached, in the untreated animal of only 4 hr. (This duration was in fact rather unusually long for a normal animal; the delay, which is subject to great variation, is more usually 1–2 hr. only.) In a second myosin experiment the delay was [illegible] hr. [illegible] Since the onset of rigor is associated with the breakdown of adenosine triphosphate (ATP) in the muscle (Bate-Smith & Bendall, 1947), it is suggested that myosin may be useful in the preparation of ATP in securing an increased yield in purification.

This work forms part of the programme of the Food Investigation Board (Department of Scientific and Industrial Research). Acknowledgement is made to the British Drug Houses Ltd. for the gift of myosin used in this work.

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Ranine ventricular dimorphism. By W. [illegible]

After sufficient perfusion the frog's ventricle is revealed to consist of two kinds of muscle, the pale and the pink. The right half is pink, the left half is pale. The two kinds of muscle differ in their physiological reactions. Pale muscle is more susceptible to the action of potassium salts than is pink, and adrenaline acts more on pink muscle than pale. Adrenaline, when strong enough, can produce a 'sore' in pink muscle.

Augmentation of output is specially associated with augmentation of effort of pink muscle.

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Insulin and blood amino-acid concentration.

By A. H. Bone and C. Reid, *Department of Physiology, London Hospital Medical College*

The R.Q. of the 24 hr. fasted cat, fed previously on a diet whose physiological R.Q. is about 0·8, is 0·74–0·76 and its O_2 usage corresponds to a metabolic rate of about 35 cal./m.^{2}/hr.

When the 24 hr. fasted cat is given sufficient insulin, e.g. 0·5 unit/kg., to cause a moderate hypoglycaemia, without obvious hypoglycaemic reactions, both the R.Q. and O_2 usage fall slightly. Further, the hypoglycaemia is accompanied by a sharp fall in the blood amino-acid concentration, but carbohydrate given by stomach tube in the form of a mixture of glucose and soluble starch at the same time as the insulin injection prevents the usual fall in the blood sugar and the blood amino-acids.

Moreover, olive oil, 30 ml. given in divided doses during the fasting period, while not preventing the fall in blood sugar, does prevent the usual fall in blood amino-acids after insulin.

The giving of oxidizable foodstuffs thus prevents the fall in blood amino-acids associated with a hypoglycaemic dose of insulin. The fall in blood amino-acids under insulin alone is secondary presumably to the fall in blood sugar and is not to be taken as exemplifying a protein inhibiting action of insulin.

The mechanical latent period of frog's striated muscle at 0° C.

By B. C. Abbott and J. M. Ritchie. *Biophysics Research Unit, University College, London*

Experiments were made to determine on a frog's sartorius at 0° C. the interval between a shock applied directly to the muscle and the earliest sign of movement. Piezo-electric recording of tension change in isometric twitches gave high sensitivity with negligible delay due to inertia. The Rochelle salt crystal ('bender') held at the ends gave an output of 0·23 V. for a tension of 1000 dynes suddenly applied in the middle, with a movement of only 2·2μ. A light chain was used in preference to an extensible thread to avoid delay in transmission: at the moment of maximum acceleration during the early relaxation (*A*, Fig. 1), the force required to accelerate the 0·64 g. of chain was only 0·25 dyne, which was negligible compared with the other forces involved. The voltage output from the crystal was passed from a cathode follower to an amplifier and cathode ray tube. The time constant of the whole apparatus was 220 msec.

The muscle was looped over a silver supporting ring attached to the recording chain, its pelvic and distal ends being tied together to a support. The ring acted as stimulating cathode obviating propagation delay. After mounting, the muscle was soaked in oxygenated Ringer's solution kept at 0° C. in a chamber immersed in ice and water; the muscle was in air during observation. The initial tension was usually 3 g. weight.

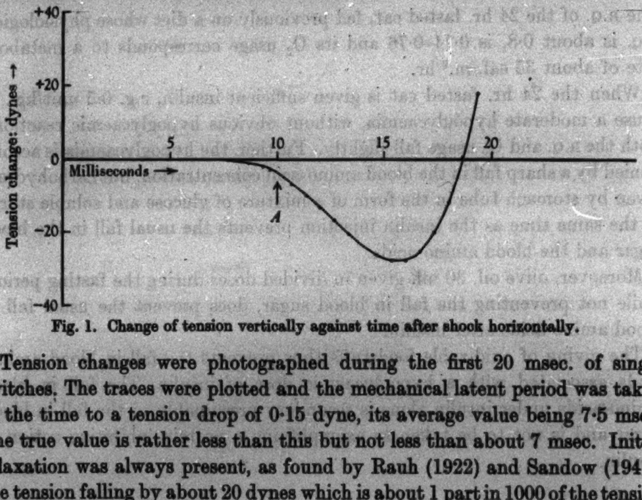

Fig. 1. Change of tension vertically against time after shock horizontally.

Tension changes were photographed during the first 20 msec. of single twitches. The traces were plotted and the mechanical latent period was taken as the time to a tension drop of 0·15 dyne, its average value being 7·5 msec. The true value is rather less than this but not less than about 7 msec. Initial relaxation was always present, as found by Rauh (1922) and Sandow (1941), the tension falling by about 20 dynes which is about 1 part in 1000 of the tension later developed. The tension changed over and became positive between 11 and 20 msec.

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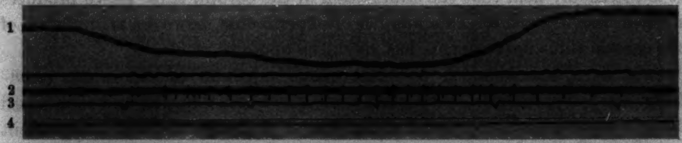
Technical aids in the study of respiratory reflexes. By R. W. TORRANCE and D. WHITTERIDGE. *University Laboratory of Physiology, Oxford*

In order to study the activity of the respiratory centre we have used a fine wire bipolar electrode introduced into the under-surface of the xiphisternal head of the cat's diaphragm, and tied within a few mm. of its tip. This electrode is made of two parallel 36–38 s.w.g. constantan wires carefully insulated and cut across obliquely at its tip. It has given records in which one unit is much larger than any other, even during powerful contraction, and the same unit has been followed continuously for 1–2 hr. This method has many advantages over the isolation of a single fibre in the phrenic nerve.

In order to separate different types of afferent fibres, the well-known methods of differential block have been used. Nerve chambers similar to those of Partridge (1939) have been cooled by alcohol circulated through a coil in a freezing mixture. In this way it is possible to cool both vagi equally and simultaneously, with thermocouple control. Compression of the vagi by sponge rubber blocks (Leksell, 1944) also gives an excellent method of blocking large fibres and leaving small fibres active. It has made the preparation of single units with a cardiovascular rhythm much less uncertain.



Record of an obstructed inspiration, showing: (1) intratracheal pressure; (2) activity of diaphragmatic motor unit; (3) E.C.G. Lead III, inverted; (4) time $\frac{1}{10}$ and $\frac{1}{100}$ sec.

These methods have given the following results. After bilateral vagotomy, the cat still shows a deflation reflex, though there is no inhibition of diaphragm activity on inflation. This deflation reflex is absent after the dorsal roots of *T*1–*T*4 have been cut on both sides.

If a vagus is cooled and activity in different single units is picked up on the headward side, stretch fibres drop out between 18 and 12° C., depressor and venous fibres about 12 to 8° C., and pulmonary capillary fibres between 8 and 4° C. When stretch fibres are blocked, the deflation reflex is still present. The 'paradoxical' contraction of the diaphragm following inflation is also present in these states of partial block. If starch granules are injected intravenously while both vagi are cooled to 6° C., rapid breathing develops. We think that these results support Hammouda, Samaan & Wilson (1943).

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Effects of electrical stimulation of the carotid sinus nerve in cats.

By E. Neil, C. R. M. Redwood and A. Schweitzer. *The Departments of Physiology, School of Medicine, Leeds, and University College, London*

It is commonly held that the stimulation of the carotid sinus nerve produces a fall in blood pressure. In a number of experiments, however, stimulation of the carotid sinus nerve in cats has given the opposite result.

Cats were anaesthetized by intravenous injection of chloralose (0·08–0·1 g./kg. body weight). The carotid sinus nerve was exposed and stimulated using a square wave electronic stimulator. This device allowed independent variation of frequency of stimuli (1·5–100 cyc./sec.), pulse duration (0·02–1·0 m.sec.) and voltage output (1–2 V.). Stimulation of the carotid sinus nerve (containing afferents from the carotid sinus and carotid body) markedly raises the arterial blood pressure. The pulse duration of the stimulus chiefly determines the degree of pressor response. Thus the threshold duration at a frequency of 100 cyc./sec. and 2 V. is 0·02 m.sec. Maximal effects (pressure increases of 60–100 mm. Hg) are mainly observed using stimuli of 0·1 m.sec. duration (Fig. 1).

Fig. 1. Stimulation of right sinus nerve. Cat, both vagi and left sinus nerve cut. Chloralose anaesthesia.

The pressor effects persist after double vagotomy, destruction of the contralateral carotid sinus nerve, excision of the cervical sympathetic, section of the glossopharyngeal nerve peripheral to the junction of the carotid sinus nerve, section of the stimulated carotid sinus nerve near its origin from the sinus and bilateral adrenalectomy. They are abolished by high spinal transection.

Stimulation of the carotid sinus nerve in the decerebrate cat or in cats under nembutal anaesthesia (intraperitoneal, 40 mg./kg. body wt.) produces the usually expected fall in arterial blood pressure, with stimuli of 0·5 m.sec. duration (100 cyc./sec. and 2 V.). Subsequent intravenous injection of $\geqq 0{\cdot}05$ g. chloralose converts this depressor response into a marked pressor one. It appears that chloralose masks the inhibitory effect of carotid sinus nerve stimulation on the vasomotor centre in cats. These responses are independent of changes in respiratory activity.

Control of cations in erythrocytes. By M. Maizels.

Department of Pathology, University College Hospital

Erythrocytes in blood stored at 4° lose K and gain Na. If such blood be warmed to 37°, glycolysis becomes active while K is regained and Na lost against the concentration gradients (Harris, 1941). It has now been found (Table 1) that gain of K increases with pH to 7·5; Na decreasing. Above pH 7·5, gain of K and loss of Na are less marked.

Table 1. K and Na in erythrocytes of blood stored with glucose, 6 days at 7° and 16 hr. at 37°. KCl added to raise plasma concentration to about 45 m.equiv./l.

		*Cell content m.equiv./l.		Final concentration in cell water m.equiv./l.		Initial concentration in plasma water m.equiv./l.		
No.	pH cells	K	Na	K	Na	K	Na	Cell glucose mg. %
†1*a*	—	61	58	85	82	47±3	170±12	312
1*b*	7·00	68	54	98	77	47±3	170±12	300
1*c*	7·51	88	30	130	44	47±3	170±12	154
1*d*	8·40	80	78	117	114	47±3	170±12	170
†2*a*	—	73	65	135	120	49±3	220±14	470
2*b*	7·34	93	36	186	72	49±3	220±14	260
2*c*	7·92	88	72	153	126	49±3	220±14	220

Bloods 1*a–d*, citrated; 2*a–c* heparinized. pH adjustment, 1*a–d* glycine-NaOH; 2*a–c*, Na_2CO_3

* Contents referred to original cell volume. † 1*a* and 2*a*, cold-stored unincubated controls.

Clearly, either Na and K both move actively against their respective gradients, or K enters erythrocytes displacing Na, or Na is actively expelled and replaced by K. If the latter, 1*d* of Table 1 with both K and Na raised might be attributed to early expulsion of Na and replacement of K followed by late readmission of Na along with external glycine and citrate (normally non-penetrating). This possibility is supported by evidence of cell damage at high pH swelling, some lysis and increased total cation.

To test if expulsion of Na is primary, non-penetrating citrate is replaced by heparin and glycine-NaOH by Na_2CO_3. If Na be not actively expelled, then, since it and external anions except protein all penetrate, cell Na should rise irrespective of K because of the preponderating osmotic effect of haemoglobin, cells swelling and lysing just as they do when placed in an ammonium chloride solution. 2*a*–*c* of Table 1 show that in heparinized blood, Na still leaves the cell. Hence, this is an active process with accumulation of K passive or independently active. In any event, it is clear that no animal cell could survive in a fluid medium without the ability to excrete Na or water.

The findings suggest that in acidosis K/Na in cells should fall and in alkalosis rise. Whether activity of glycolysis decides the varying K/Na ratios of animal erythrocytes remains to be determined.

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Sympathin, histamine and acetylcholine in mammalian nerves.

By U. S. v. EULER. *Department of Physiology, Karolinska Institutet, Stockholm*

Extracts of adrenergic nerves contain considerable amounts of a sympathomimetic substance which conforms in its actions and properties with *nor*-adrenaline or sympathin (Euler, 1946*a*, *b*). Splenic nerve extracts from cattle exert, in addition to the pressor action, strong depressor activity on the cat's blood pressure after atropine. This action corresponded to that of histamine on the cat's blood pressure and on the guinea-pig's intestine before and after atropine and antihistaminic substances. The active extracts also gave a positive Pauly reaction, roughly corresponding to the amount of histamine found biologically. This was equivalent to 60–120 μg. histamine dihydrochloride/g. of fresh splenic nerves, freed from their sheath and prepared 1–4 hr. after the death of the animal.

TABLE 1

Nerve (cattle)	Acetylcholine (μg./g.)	Histamine (μg./g.)	*dl-nor*-Adrenaline (μg./g.)
Optic nerve	<0·02	3–10	<0·5
Ciliary (postganglionic cholinergic)	3–8	10–30	0·8
Splenic (postganglionic adrenergic)	0·2–0·5	60–120	10–25
Sympathetic chain, fibres	3–8	30–80	3–5
Sympathetic chain, ganglia	3–6	40–80	2·5–5
Cutaneous sensory nerves	0·1	25–40	1
Phrenic	2–3	20–30	<0·5

In extracts of various nerves, made with acid alcohol or trichloroacetic acid, biological determinations were also made of the amount of *nor*-adrenaline and acetylcholine equivalents (cf. Lissák, 1939) in order to obtain what may be termed an 'ergone spectrum' of the nerve. The figures in Table 1 are given in equivalents of the hydrochlorides of the substances of comparison in μg./g. of fresh nerve.

The large differences in the histamine content suggest some specific action of this ergone, correlated to the function of the nerve as is known for acetylcholine and sympathin.

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Plasma protein determination in rats using the copper sulphate method. By S. E. Dicker.[*] *Department of Pharmacology, University of Bristol*

Moore & Van Slyke (1930) showed that the total plasma proteins can be calculated from the specific gravity by the formula $P=K(S-A)$, where K and A are constants for which they assumed the values of 343 and 1·007 respectively. This equation has been used to estimate the plasma protein concentrations of human beings by means of the 'copper sulphate method' (Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald, 1945). Phillips *et al.* (1945) proposed different values for K and A, viz. 389·6 and 1·0079. These values were criticized by Hoch & Marrack (1945), who showed that in human beings the equation giving results nearest to the value obtained by a Kjeldahl estimation of the nitrogen of the plasma was $P=364\,(S-1{\cdot}006)$.

Hoch & Marrack's (1945) equation has been used to estimate the plasma-protein concentration of normal rats and of hypoproteinaemic rats fed on a low-protein diet (Dicker, Heller & Hewer, 1946). Plasma nitrogen was determined by a micro-Kjeldahl method. The samples were digested with SeO_2 for 5 hr. (*Report of the Advisory Committee of Nutrition Surveys of the Nutrition Society*, 1945).

Table 1

		Plasma proteins (g./100 c.c.)		
	Series	Kjeldahl	Specific-gravity method	't'
Normal rats	A	7·31±0·08 (16)	7·34±0·07 (16)	0·687, $P>0{\cdot}4$
	B	7·03±0·11 (11)	7·03±0·09 (9)	0·007, $P>0{\cdot}9$
Rats with experimental hypoproteinaemia	C	6·70±0·15 (9)	6·74±0·13 (9)	0·636, $P>0{\cdot}5$
	D	5·11±0·14 (29)	5·21±0·13 (29)	0·477, $P>0{\cdot}6$
	E	4·65±0·10 (10)	4·70±0·09 (10)	0·392, $P>0{\cdot}7$

The values are means and standard error. In brackets, number of rats.

Table 1 compares plasma-protein values obtained by micro-Kjeldahl estimations with values obtained by the specific-gravity method using Hoch & Marrack's (1945) equation. It will be seen that the two methods yielded comparable data, there being no significant difference between the mean results. The equation proposed by Phillips *et al.* (1945) was also applied, but the values obtained were significantly different from those obtained by the micro-Kjeldahl estimation.

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[*] Beit Memorial Fellow.

Provided Hoch & Marrack's (1945) equation is used it can therefore be concluded that the copper sulphate method permits quick and reliable estimation of the plasma protein concentration in rats.

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A correction to the effect of temperature on the activity of carbonic anhydrase. By F. J. W. Roughton

The activity of carbonic anhydrase has usually been determined manometrically from the rate of CO_2 output from phosphate-bicarbonate mixtures, pH 6·8–7·8, temperature 0–15° C. in presence and in absence of the enzyme. For the non-enzymic rate, Meldrum & Roughton (1933) gave Q_{10} (= temperature coefficient per 10° C. rise) = 3·6, and for the enzymic rate $Q_{10} = 2{\cdot}1$. These values have been generally used in calculating the effectiveness of the enzyme at 38° C. Lately, I have discovered that these Q_{10} values are both too high, owing to errors in working out the original data.

As a measure of the rate of the reaction we took the reciprocal of the time for a given change in level of the manometric gauge fluid, when the CO_2 output is between one-quarter and one-half complete. If the non-enzymic rate at 0·2° C. is taken as unity, the new corrected rates are

Temperature in ° C.	0·2	19·2	25·05	29·95	34·95
Non-enzymic rate	1	9·7	15·7	28·2	41·5
Enzymic rate	12	22·8	29·3	—	43·5

These data give an average Q_{10} for the non-enzymic rate = 2·9, and for the enzymic rate (i.e. overall rate minus non-enzymic rate) = 1·4, over the range 0–35° C. The 'magnification' of the rate of CO_2 output by the enzyme concentration used in this work drops from 12 at 0·2° C. to 1·05 at 34·95° C. The logarithms of the respective rates, when plotted against 1/absolute temperature, give points falling practically on straight lines. This suggests that the great violence of shaking in these experiments was sufficient to prevent diffusion from limiting appreciably the rate of CO_2 output even at 35° C.

Recalculation with the above figures shows that the enzyme content of the normal human red cell* should magnify the rate of the non-enzymic reaction at 38° C. by about 5000-fold, which is within the range of 1500- to 7500-fold given previously (Roughton, 1943). All such estimates disregard possible complications within the red cell from the enormously higher enzyme concentration, the different environment, and the limiting effect of diffusion. Unpublished calculations of mine, based on Dirken & Mook's (1930) data on the rate of CO_2 uptake by red cells and strong Hb solutions, make it doubtful, however, whether an error > five-fold at most is caused by such disregard. In a forthcoming paper Davies and I have applied the revised Q_{10}'s to the calculation of the enzyme activity in the oxyntic cells of the stomach.

* It is assumed that the red cell contains about 4 Meldrum-Roughton units per cu.mm.

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Some adaptation effects using yellow stimuli. By H. Hartridge.

Vision Research Unit (*Medical Research Council*)

Edridge-Green & Marshall (1909) stated that they found definite evidence for a separate retinal receptor for yellow rays. To test this view, three colour filters were prepared, one transmitting a lemon-yellow light which consisted spectroscopically of a narrow band which centred on 0·57μ; another transmitted a yellow light which consisted of two bands, one in the red centring on 0·69μ, and another in the green centring on 0·53μ; and a third wide-band filter transmitted nearly the entire spectrum, except for the violet and part of the blue. These three filters were carefully adjusted so that the rays which passed through them to the eye were as nearly as possible alike in hue, saturation and brightness.

After exposure to light through any one of the filters a spectrum, produced by a small hand spectroscope, was quickly examined and was seen to be darker than usual. Moreover two of the filters were found to produce specific effects in addition. Thus after exposure for 1 min. through the narrow-band monochromatic filter the blue part of the spectrum was considerably darkened, the red region had a purplish pink colour, the green appeared pale blue-green, and the yellow region in between them looked neutral grey. On continuing to watch the spectrum the yellow slowly reappeared, and the red, the green and the blue regions regained their normal appearance. After exposure through the dichromatic filter the specific effects are much less marked, and after exposure through the wide-band filter they are hardly noticeable.

On Young's theory it would be anticipated that the monochromatic and the dichromatic filters would produce the same effects, except perhaps that the latter, since it transmits green rays, might be expected to stimulate the blue receptors and so to produce more fatigue than the monochromatic does. On the same grounds the wide-band filter would be expected to affect the blue receptors with fatigue even more than the other two filters. But in fact the monochromatic filter has the most effect on the blue, and the wide-band the least.

So far as the yellow region is concerned it would be expected on Young's theory that the monochromatic and the dichromatic filters would have the same effect, since both stimulate the red and the green receptors. But experiment shows that the magnitude of their effects are quite different, for the light through the monochromatic filter causes yellow to be replaced by grey, whereas the dichromatic filter has hardly any effect on yellow. It is concluded that there must be specific receptors for yellow rays, which are independent of those for red rays and green rays, and that these yellow receptors are affected by monochromatic yellow light, but only slightly by yellow lights of different spectral composition.

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The spontaneous movements of the rat diaphragm. By I. Hajdu, R. J. S. McDowall and A. Z. Shafei. *King's College, University of London*

In a previous communication (McDowall & Shafei, 1946) evidence was put forward which suggested that resting muscle synthesized acetylcholine. This view is now extended. If the rat diaphragm is kept in a modified Tyrode solution in which calcium chloride is reduced to 0·01% and glucose to 0·0025% the muscle exhibits twitches similar to those produced by eserine, and often a contracture. Like eserine twitches they are abolished or reduced by rapid or strong stimulation of the muscle through its nerve by calcium and by glucose above 0·2% and by curari. A minute amount of glucose is essential for their production. Their production is associated with the transfer of some substance from the muscle to the bath for they may be abolished for a short time if the fluid in the bath is replaced by fresh Tyrode solution of similar composition and after repeated washings may fail to appear. They are at first enhanced by the addition of choline and adrenaline which are known to aid acetylcholine synthesis, but later are depressed by adrenaline.

This and the abolition of twitches by calcium and glucose which depress such synthesis suggest that twitches are due to the accumulation of acetylcholine, but if this is so the accumulation must take place in such a position or way that it cannot be destroyed by the cholinesterase present.

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A soap bubble volume recorder. By A. D. M. Greenfield. *From the Physiology Department, St Mary's Hospital Medical School, London*

This volume recorder has been developed to record rapidly changing small volumes, while exerting a minimum of restriction on the system being measured. The method is essentially the same as that of Straub (1910). A full examination of the properties of bubble recorders of different dimensions has not been made, and the following remarks apply to one developed for a specific purpose.

A soap film is spread across a horizontal circular orifice 35 mm. in diameter, which is at the top of an elongated cone, the lower small end of which is connected by a wide bore rubber tube of the shortest possible length to the plethysmograph. This bubble is suitable for volume changes up to 5 c.c., and in use is arranged to travel between slight convexity and a convexity in which its centre is raised about 10 mm.

A light beam from a condenser is passed tangentially across the bubble, and an image of the apex of the bubble and a cross wire base line is cast on to moving photographic paper by an achromatic lens. The shadow of the bubble forms a surprisingly sharp image.

Static calibration shows almost complete linearity over the range used. Dynamic calibration with a 3·5 c.c. excursion of simple harmonic motion shows an increase in amplitude of the record of 1% at 700 cycles per minute, 5% at 1100 cycles and 10% at 1500 cycles. The bubble becomes conical at the higher speeds, and fluid is drawn into the centre, where it may be thrown off as droplets. The shape may be due partly to resonance.

The pressure within the bubble increases as it is inflated, and the radius of its curvature decreases. At 5 c.c. of filling, the pressure within the soap bubble is less than $\frac{1}{2}$ mm. of water.

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A simultaneous study of the action potentials and movements of single motor units in the tonic stretch reflex.

By G. Gordon and A. H. S. Holbourn. *Laboratory of Physiology, University of Oxford*

Apparatus has already been described for simultaneously recording action potentials and movements from single units in small human muscles, using a pair of electrodes and a piezo-electric system (Gordon & Holbourn, 1948). This has now been modified for recording from superficial units in the intact crureus of the decerebrate cat, picking up from the surface of the muscle and recording longitudinal movements of the fibres.

Several authors have previously noticed double or multiple electrical discharges occurring in a single unit, especially at the beginning of stretch and crossed extensor reflexes: as the contraction proceeds, the usual single discharges appear, with an increase in the frequency of rhythmic firing.

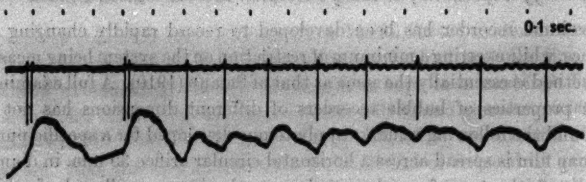

Fig. 1. Action potentials (top line) and movements (bottom line) from a single unit of crureus during a slight progressive stretch of the muscle. (Action potentials retouched to make line drawing.)

When single and double discharges are observed in a stretch reflex together with their mechanical counterparts (Fig. 1), it is clear that the pull of the motor unit for a double discharge is two or more times that for a single discharge, there being a complete fusion of the two twitches. Such a mechanism provides very swift increments of tension at the beginning of a reflex contraction, as Hoff & Grant (1944) have suggested. This must incidentally be one of the few occasions when optimal mechanical fusion occurs in a single motor unit.

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Pyridoxin in relation to amine formation in the mammalian liver.

By H. Blaschko, C. W. Carter, J. R. P. O'Brien and G. H. Sloane Stanley

Determinations have been made of the dopa decarboxylase and cysteic acid decarboxylase activities of pyridoxin-deficient rats. The codecarboxylase content of the liver of such animals has also been assayed.

The animals were maintained on a basal diet of casein 19 %, sucrose 67 %, salts 5 %, cod-liver oil 4 % and water 5 %, together with vitamin B_1 20 μg., calcium *d*-pantothenate 240 μg., *p*-aminobenzoic acid 250 μg., nicotinamide 10 μg., inositol 500 μg., and choline chloride 1 mg. daily. The procedure of paired feeding was adopted; the controls received 40 μg. pyridoxin daily in addition to the other vitamin supplements.

Most of the deficient animals showed mild or severe dermatitis, varying with the duration of depletion.

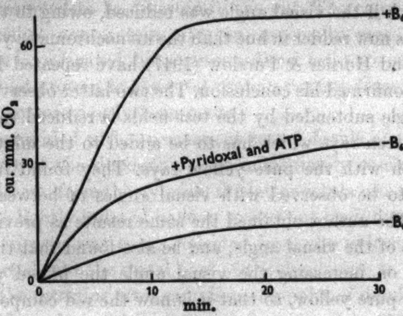

Fig. 1.

The Table indicates a marked decrease in the dopa and cysteic decarboxylase activity of extracts of livers of deficient animals as compared with controls. There is also a decrease in the liver content of codecarboxylase in the deficient animals.

Dopa q_{CO_2}		Liver codecarboxylase as % of control	Cysteic acid q_{CO_2}		Liver codecarboxylase as % of control
Control	Deficient		Control	Deficient	
0·79	0·14	—	0·21	0·0	—
0·48	0·07	—	0·20	0·06	—
0·71	0·13	—	0·13	0·0	—
0·81	0·0	—	0·15	0·01	50
0·38	0·14	—	0·15	0·0	29
0·86	0·17	—	—	—	—
0·99	0·17	45	—	—	—
0·59	0·11	68	—	—	—
0·56	0·07	48	—	—	—
0·62	0·07	33	—	—	—

Addition of pyridoxal plus ATP raised the dopa decarboxylase activities of some liver extracts from deficient animals, though the activity was never wholly restored to the level of the controls (Fig. 1). In some cases no reactivation occurred, suggesting disappearance of the apo-enzyme as a result of depletion. No reactivation has so far been obtained with cysteic acid decarboxylase.

These results suggest that in mammals, as well as in bacteria, amine formation is brought about by enzymes which contain a derivative of pyridoxin.

The effects of alteration of visual angle on the perception of yellow stimuli.

By H. HARTRIDGE. *Vision Research Unit, Central London Ophthalmic Hospital, Judd Street, London, W.C.* 1

Hering (1893) produced matches between monochromatic yellow rays and suitable mixtures of red rays and green rays. He found that a match made when the test objects subtended a given angle at the eye of the observer was no longer a match if the visual angle was reduced, owing to the fact that the mixed yellow was now redder in hue than the monochromatic yellow. Recently Shaxby (1947) and Horner & Purslow (1947) have repeated Hering's experiment and have confirmed his conclusion. The two latter observers showed that as the visual angle subtended by the test fields is reduced the greater is the amount of the green rays which has to be added to the mixture in order to preserve a match with the pure yellow rays. They found further that the phenomenon is to be observed with visual angles of between one and five degrees of arc. The author obtained the same results as previous workers on reducing the size of the visual angle, and he also found that the reverse effect takes place, for on increasing the visual angle the mixed yellow becomes greener than the pure yellow, so that it is now the red component which has to be increased in the mixture in order to preserve a match. This phenomenon is found to continue up to at least twenty-five degrees of arc. This increase in the green at larger visual angles and decrease in the green at smaller ones might be explained on the hypothesis that the foveal pigment absorbs green rays but not yellow or red, and that this pigment increases progressively in density from the periphery to the centre of the fovea. If this were the true explanation it would be expected that a yellow-green would alter to yellow as its visual angle is reduced in size, but in fact it alters to blue-green, and this proves this hypothesis to be untenable. According to the three-colour theory a mixed yellow and a pure yellow which match one another at one visual angle should also match at another, but since they do not do so, this result is at variance with the three-colour theory. On the contrary it is in favour of the view that there are independent yellow receptors, because the red-green mixture can become either redder or greener, with change of visual angle, without causing a corresponding alteration in the hue of yellow. The other changes in hue which are found to occur as the visual angle is reduced are also in favour of the latter hypothesis.

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The extent of conversion of food protein to microbial protein in the rumen of the sheep. By Ian W. McDonald. *The School of Biochemistry, University of Cambridge.* (Introduced by A. T. Phillipson)

The micro-organisms which grow prolifically in the rumen utilize nitrogen in the animal's food for the formation of their own protein. The extent to which this process occurs is important as it undoubtedly influences the biological value of the protein passing to the abomasum and the small intestine. Microbial protein is assumed to be digested in the gut. The capacity of the micro-organisms to utilize non-protein nitrogen, especially urea and amides, has been extensively investigated, but even so a direct estimate of the contribution made by microbial protein to the total protein passing from the rumen to the abomasum has not previously been made.

A sheep was provided with a permanent rumen fistula and two fistulae into the anterior part of the duodenum, one immediately caudal to the pylorus and one about 10 cm. from the pylorus; the latter fistula was used to insert into the duodenum a lightly inflated balloon to prevent back-flow of the intestinal contents while ingesta was being collected from the pyloric fistula. A third fistula was inserted into the distal part of the duodenum to enable ingesta to be returned to the intestine after sampling. Samples of duodenal ingesta were collected from the pyloric fistula before and at intervals after feeding.

The sheep was fed a diet in which zein represented 82% of the total protein. The daily intake of food was constant. Zein contains no lysine and is soluble in alcohol. The ruminal micro-organisms were found to contain no alcohol-soluble protein and their mixed proteins contained about 7%. lysine. These characteristics made it possible to calculate, on the basis of estimations of lysine or alcohol-soluble protein in the duodenal ingesta, the fractions of the total protein leaving the stomach represented by microbial protein and by zein.

In preliminary experiments it has been estimated that about 40% of the zein in the diet was converted in the rumen into microbial protein.

A method of measuring the flow of digesta from the stomach of the sheep. By A. T. Phillipson. *The Rowett Research Institute, Aberdeenshire*

The passage of digesta from the stomach of the ruminant is said to be continuous although there is little direct experimental evidence to support this statement, neither are there any data to show the volume of digesta leaving the stomach in a given time.

The following method was developed to supply this information and is now being used to study digestion in the stomach on a quantitative basis. A cannula, made of perspex, is inserted into the duodenum adjacent to the pylorus without serious obstruction and the sheep is allowed at least a fortnight to recover from the operation. Two more cannulae are then inserted at a subsequent operation, close together, into the caudal end of the duodenum.

When the first cannula is opened digesta flow from it at irregular intervals. The second cannula is also kept open and usually the flow from it is scanty, the material consisting principally of bile and other secretions. If, however, the first cannula becomes blocked, digesta flow from the second cannula in quantity. This flow ceases when the first cannula is cleared and it is essential to collect from both the first and second cannula if a true measure of the digesta flowing along the duodenum is to be obtained. The remaining cannula is used to reintroduce digesta into the intestine after they have been collected, measured and sampled. Temporary inflation of a small balloon inserted into the duodenum through the second cannula prevents back flow.

Quantities of digesta collected for 1½ hr. from the first and second cannulae of a well-trained sheep at different times throughout the day show that there is no simple relation between the total dry matter of the food and the flow of digesta and suggest that the nature of the food—possibly its protein content—increases the volume of digesta flowing from the stomach.

Flow of digesta per hour

No. of collections	Range (ml.)	Mean (ml.)	Total dry matter of the food (g.)	Food
4	643– 889	798	868	2¼ lb. hay in 3 meals
5	890–1157	1038	1735	4½ lb. hay in 3 meals
9	1504–1864	1608	1738	4 lb. hay; ½ lb. linseed meal 2 meals of hay and 1 of meal

The average volume of water drunk during the period of these observations was a little over 3 l. a day, the greatest quantity being 5·4 l. In so far as these collections cover the period from 9 a.m. to 5 p.m. the digesta flow in large or small gushes from the cannula every few minutes and it is unusual for the interval between the gushes to exceed 15 min. The total amount passed in 1 hr. is subject to considerable variation, the nature and quantity of the food exerting an important influence.

The operative procedures did not appear to have an adverse effect on the well-being of the sheep concerned in these experiments for its weight has increased since the initial operations were performed.

Some uses of a conductivity cannula in the cat.

By J. A. B. Gray and W. D. M. Paton. *National Institute for Medical Research, London, N.W. 3*

Stewart (1894) measured the circulation time in dogs by injecting hypertonic NaCl and recording its arrival at a distant vessel with a pair of electrodes and a resistance bridge. We have made similar measurements in chloralosed cats with a cannula tied in continuity with suitable vessels. The cannula contains three silver electrodes equally spaced along its length, the outer two being shorted to each other, and forms one arm of a parallel capacity 1000 cyc./sec. bridge, the balance of which is recorded with amplifier and oscillograph on moving paper. This method is sufficiently sensitive to allow 0·9% NaCl to be used as the indicator. The ordinate of the records can be calibrated with an error of <10% and the volume of indicator between the electrodes at a given moment can be expressed as a percentage of the total effective volume of the cannula. If the flow through the cannula (vol./time) is known, the time-scale can be converted to a volume and the area of the tracing represents the volume of indicator (cf. White, 1947).

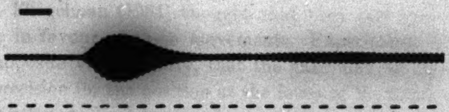

Fig. 1. Record from conductivity cannula in carotid artery showing 1st and 2nd circulations of 3 ml. of saline injected into femoral vein during signal. Time, 1 cycle = 1 sec.

Taking times from the beginning of a brief injection to the peak of the response, we have obtained the following mean values: femoral vein to carotid or aorta 6·3 ± 0·32 sec. (S.E. of mean of 13 cats); femoral vein to carotid or aorta (second time round) 18·2 ± 1·43 sec. (8); aorta to inferior vena cava 12·5 ± 1·03 sec. (3); aorta to carotid or aorta 14·1 ± 1·30 sec. (8). No single figure, however, can define the 'circulation time'; the curves may be taken as equivalent to the frequency distributions of the times of arrival of a population of red cells leaving a distant point at a given moment, and the most accurate measure of blood circulation time would be to give enough parameters to define these curves.

Concentrations of injected drugs in arterial blood can also be estimated by this method. Thus, with histamine dissolved in 2–3 ml. saline and injected in 1–2 sec. into the femoral vein, the peak concentration of histamine base for a 10% fall of blood pressure is $1\text{–}5 \times 10^{-9}$, and the fall begins about the time of the peak concentration. In general, for such injections, the peak arterial concentration is 2–10% of the concentration of the solution injected intravenously.

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PROCEEDINGS
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A method of investigating eye movements. By H. Hartridge and L. C. Thomson. *Vision Research Unit, M.R.C.*

Hering (1899) thought that if a fixed pattern existed on the retina a straight line would be seen as broken and irregular, and that these defects are avoided by chance eye movements. Many other physiologists have held similar views (Anderson & Weymouth, 1923; Averill & Weymouth, 1925; Marshall & Talbot, 1942). Adler & Fliegelman (1934) thought that they had obtained experimental evidence in favour of these movements. Experiments by one of us supported the opposite view, namely, that the gaze may be held stationary with sufficient precision for the position at the fovea of the fixation points for rays of different colour to be determined.

The demonstration shows the apparatus which has been devised by us in order to investigate small rotations of the eye. The image of an ophthalmoscope filament lamp reflected in the cornea of the subject is projected by means of a lens system consisting of a low-power microscope objective and field lens. This lens system converges the image on to the focal point of a collimating lens, the rays from which are received either by a ciné camera or by the observer's eye via a prism monocular. In the former case the film is subsequently measured by means of a micrometer microscope.

The projection lens system is attached to a steel and plaster cap which accurately fits the head of the subject. Also attached to the plaster cap is a small reference lamp. An image of this lamp is arranged to lie at the focal point of the collimating lens, so that both it and the image reflected from the cornea are seen side by side. Thus rotations of the eye will cause relative movements between the reference and the corneal images. Movements of the head, on the other hand, will cause *both* images to move together on the ciné film. Consequently by the use of this method rotations of the eye may be measured in spite of the presence of head movements, which might otherwise disturb the observations. The camera is mounted on an optical bench, which is independent of the projection lens system in order to avoid the transmission of vibrations to it.

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Pipette-type Geiger counter for small quantities of biological fluids.

By D. M. Maurice. *University College, London*

This counter was designed in connexion with investigations of the penetration of hard β-ray-emitting tracers into the intra-ocular fluids of the cat, and had to meet the following requirements: (1) use with small liquid samples of 0·5–1 ml.; (2) good reproducibility in the positioning of the sample with respect to the counter; (3) recovery of the sample unchanged, for further study.

It consists (Fig. 1) of a conventional cylindrical glass-walled counter, with a thin central window round which is sealed a glass cylinder, forming an annular space about 1 cm. wide and 0·5 mm. deep. Capillary tubes are fixed in the top and bottom so that the space may be filled with the sample by suction; just under 0·5 ml. is necessary.

Requirement (2) is evidently satisfied; no trouble has been experienced with air locks or bubbles provided that ordinary care was taken in filling.

On blowing the sample out, just over three-quarters are recovered. A fairly constant fraction remains behind, and use may be made of this property in order to avoid cleaning and drying the counter between samples if only about 5% accuracy is aimed at. The residue of the old sample may be allowed to contaminate the new and an appropriate correction made, or, alternatively, the counter may be washed out with water, the diluent effect being equal on all samples.

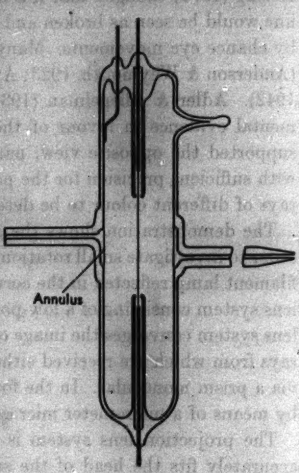

Fig. 1.

If greater accuracy is required, or contamination of the sample is not permitted, the counter must be washed and dried. The procedure used has been successive rinsing with water, alcohol, ether and ether containing a trace of caprylic alcohol, followed by removal of the ether in a current of air. The counter can be emptied, cleaned and refilled within 3 min.

A d.c. amplifier with alternative a.c. coupling.

By P. O. Bishop and E. J. Harris. *Department of Anatomy and Biophysics Research Unit, University College, London*

An amplifier is used, with a gain of over 10^6, for studying resting potentials and 'slow' waves in brain cells.

An ergometer for myothermic experiments. By A. V. Hill. *Biophysics Research Unit, University College, London*

This ergometer was designed to avoid the dissipation of mechanical energy in the muscle when it relaxes, and—with the appropriate load—to obtain the greatest possible work. The force to be overcome diminishes as the muscle shortens, the weight finally tips over and the muscle is left unloaded. The load and the distance lifted are adjustable: the work done is their product.

An equipment for the display and recording of physical changes associated with physiological events. By V. H. Attree. *Biophysics Research Unit, University College, London*

The use of an appropriate 'pick-up' enables certain physical changes (e.g. mechanical, thermal, optical, etc.) to be transformed into voltage changes which may be displayed on a cathode-ray tube. The equipment amplifies the electrical signals from the 'pick-up' and provides a display which is normally recorded on a fixed plate camera. A double-beam cathode-ray tube is used enabling two signals to be recorded simultaneously. In the demonstration given the signal on one trace records the heat production in a muscle, and on the other trace the associated mechanical movement. 'Pick-up' units for changes of pressure, temperature, velocity, acceleration and force have been used in this laboratory, but it is possible to record changes in many other physical quantities.

The measurement of pressure by the change of temperature in the adiabatic compression of oil. By A. V. Hill. *Biophysics Research Unit, University College, London*

The temperature of any substance with a positive coefficient of thermal expansion rises reversibly during compression. Paraffin oil has a large coefficient and its temperature rises about 0·012° C./atm. The temperature change can be measured with a 'thermistor'. The instrument shown has been used to determine the pressure developed (100–300 mm. Hg) inside a frog's gastrocnemius when it contracts.

Counting and scaling apparatus. By E. J. Harris. *Biophysics Research Unit, University College, London*

This equipment, constructed in the laboratory apart from the scaler, is used for the measurement of radioactivity. Radioactive isotopes of potassium and sodium are being applied to a study of the kinetics of the penetration or adsorption of these ions in muscle.

A mass spectrometer (Nier type). By E. J. Harris. *Biophysics Research Unit, University College, London*

This equipment, constructed in the laboratory, is for using the stable heavy isotopes of carbon and nitrogen to study the metabolism of compounds of biological importance.

Response of the phrenic nerve of the rat to rectangular pulses of direct current. By G. A. Mogey and J. W. Trevan. *The Wellcome Physiological Research Laboratories*

Just supra-maximal stimulation (1–2 V.) of the isolated phrenic nerve in Ringer's solution (Bülbring, 1946) with rectangular pulses of d.c. shorter than about 1·26 msec. does not produce maximal contractions of the diaphragm. Increasing the duration of the stimulus above 1·26 msec. produces progressive increase in size of contractions of the diaphragm until the duration reaches about 50 msec. which gives a 'twitch' equal to that produced by a stimulus of 'infinite' duration. The increment in contraction takes place at first in steps. In one experiment sudden increments took place at 1·26, 1·40 and 1·58 msec., the height of contraction for durations of stimuli between the steps remaining constant. In some experiments no steps are apparent, the height of contraction increasing more or less smoothly, with smooth equal increments of duration of stimuli above about 1·26 msec. The phenomenon persists for many hours after setting up the preparation, although the height of the twitches produced by the longer stimuli may diminish.

It is unlikely that the effect is due to the existence of fibres of differing chronaxie, for the size of chronaxie required would exceed any that have been reported by an enormous margin. The most probable explanation is that the longer-duration stimuli produce a repetitive response in the nerve fibres, and the corresponding muscle 'twitches' are, in fact, short tetani. Where the increment in contraction is stepwise, the rate of repetitive response is synchronous in the majority of the fibres. Asynchronism leads to a gradual and not a stepwise increase in height of contraction. The maximum number of steps observed was four at durations lower than about 6 msec., so that asynchronism always sets in at about this duration. *d*-Tubocurarine abolishes the difference between the effects of long and short stimuli in concentrations too low (about 1×10^{-6}) to diminish the height of the response to short stimuli. This corresponds to the known fact that the tetanic response of a muscle to rapidly repeated nerve impulses is converted by *d*-tubocurarine into a single twitch. Eserine (1×10^{-8}) increases the response of the muscle to a stimulus of less than 1·26 msec., and the effect is abolished by minute doses of *d*-tubocurarine (2×10^{-8}). The eserine presumably produces a repetitive response of the nerve ending to a single impulse in the nerve fibre.

The occurrence of repetitive responses of nerve fibres has often been reported before, but usually as occurring under special conditions (e.g. Erlanger & Blair, 1936). It seems to be the normal response of the isolated rat and mouse phrenic nerves to stimuli of sufficient duration,

We are indebted to Dr G. L. Brown for much stimulating discussion of our results.

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Heat production in a muscle twitch. By A. V. Hill. *Biophysics Research Unit, University College, London*

The heat production in a muscle twitch begins at the same moment (within 1 or 2 msec. at 0° C.) as the mechanical response. It starts off at its maximum rate and finishes as contraction ends. In relaxation no heat appears unless mechanical work is dissipated in the muscle. There is no sign of heat absorption at any stage in contraction or relaxation. With varying load (from constant initial length) the work done may vary from 0 to 50% of the heat, but the heat is constant and independent of the load and work. Clearly the stimulus does not fire a 'cartridge' containing constant energy. Can heat and work be derived from different sources?

When a muscle relaxes without load it remains shorter; this can scarcely be due to folding of the fibres because, stimulated in the shorter condition, it produces much less heat than if gently pulled out beforehand.

The maximum work in the single twitch of a frog's sartorius at 0° C. is sufficient to lift the muscle 60–70 cm. against gravity; this is at least as great as at a higher temperature. The mechanical efficiency of the initial process may be as high as 35%. The recovery heat at 0° C. is about equal to the initial heat plus work, as at other temperatures.

If excitation be assumed to occur at the surface of a fibre, diffusion inwards is far too slow to account for the speed at which contraction can develop. Some physical change, not a chemical substance, must be propagated in.

These facts tend to be disregarded in current speculations about the chemistry of contraction.

Central summation in vasodilator and respiratory reflexes. By H. Rosenberg. *Department of Physiology, Royal Veterinary College, London*

In order to apply repeated stimulation of variable but identical rates, simultaneously to two nerves, at a reproducible strength predetermined independently for each nerve, the output of a square wave oscillator was fed, with a 2 μF. capacitor in series, into the primary of a stepdown transformer whose secondary was connected with the primary coils of two matched inductoria in parallel.

The strength of the stimulation of either nerve was adjusted, while the secondary coil of the opposite inductorium was shunted by a non-reactive resistor, approximately equivalent with the nerve resistance to 0·1 msec. rectangular pulses. Under these conditions the shocks are non-oscillatory and reach the peak in 0·1 msec. on make and in 0·09 msec. on break (fall to 30% of maximum in 0·35 and 0·31 msec. respectively). The shock voltage on make is about 10% higher than on break. As the bilateral shocks are synchronous, a coincident arrival of the afferent impulses at the medullary half-centres may be presumed.

Unilateral stimulation of the aortic nerve (rabbit under urethane, both aortic nerves, sympathetics and vagi cut, both carotid sinuses excised) shows temporal summation of vasodilatation, e.g. no response at 4 and clear fall of arterial pressure at 30 double shocks per sec. (make-break interval 12 msec.). In the visceral effectors concerned, temporal summation in part probably is peripheral (Wright, 1928), while the increase in respiratory rate observed under these conditions cannot be assigned to the somatic effectors.

On bilateral subthreshold stimulation, a fall of arterial pressure, or an increased fall when the strength on one side only is subliminal, points to spatial summation in the, presumably linked, vasodilator half-centres (summation in afferents evidently ruled out). If stimulation of both nerves is supraliminal, a fall in excess of addition of the separate responses seems indicative of true spatial central summation, actually observed in the vasodilator reflex response. In the respiratory reflex response, increase in rate above addition was found while ventilation did not exceed partial addition (respiratory responses uncertain or absent in many animals). Gesell, Hansen & Siskel (1947) obtained similar results on stimulation of the carotid sinus nerves in dogs.

Spatial addition and temporal summation were also found in vagal respiratory inhibition (rabbit, same conditions). Rebound often was marked.

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A double histamine test for inhibitors of gastric secretion. By Henry T. Howat and B. Schofield. *Department of Physiology, Manchester University*

In fasting chloralosed cats a tube is passed into the stomach from an opening in the oesophagus, the pylorus ligated, and the stomach washed out with warm water. Atropine sulphate (1 mg./kg.) is administered intravenously and the rectal temperature kept constant.

After approximately 1 hr., the stomach is washed with N/200-HCl, and 25 c.c. are left in. This is renewed every 15 min. and the free and total acid of the samples estimated by titration with N/20-NaOH. The results are plotted as output of N/10-HCl per 15 min. period. Histamine acid phosphate in 0·9% NaCl is administered intravenously at a constant rate of 0·0045 mg./kg./min. (0·00155 mg. histamine base) for 45 min. from a motor-driven syringe. The acid secreted constitutes the first response. After the acidity has returned to the basal level, the 45 min. histamine injection is repeated, producing the second response. The test substance is administered intravenously between the two injections.

A series of seventy-six experiments show a mean first response of 10·20 c.c. total acid (S.E. of mean ±0·44 c.c.). The difference between the first and second responses is expressed as a percentage of the first, and in a series of experiments the mean of these percentage differences is calculated, this being described as the *mean % diff.* A control series of twenty-five experiments with no intervening injection show a *mean % diff.* of −10·8±3·57.

Tests have been performed on the following substances:

(1) *Urogastrone* prepared by the method of Gray, Wieczorowski, Wells and Harris (1942), and tested in the crude and purified forms;

(2) *Enterogastrone concentrate* prepared as described by Greengard, Atkinson, Grossman and Ivy (1946);

(3) *Neo-antergan* (Anthisan M. & B.) as an example of the synthetic anti-histamine drugs.

Significance was tested by Fisher's *t* test (Fisher, 1946).

Substance	Dose	No. of exps.	Mean % diff.	S.E. of mean	Significance of variation from mean % diff. of controls
Urogastrone, crude	10–25 mg.	11	−80·3	± 4·12	$t=11{\cdot}5$, $p<0{\cdot}01$ Highly significant
Urogastrone, purified	1–6 mg.	8	−49·1	± 9·68	$t=4{\cdot}6$, $p<0{\cdot}01$ Highly significant
Enterogastrone concentrate	1 dog unit	3	−69·7	±13·39	$t=5{\cdot}3$, $p<0{\cdot}01$ Highly significant
Enterogastrone concentrate	<1 dog unit	5	−17·0	± 9·65	$t=0{\cdot}69$, $p=0{\cdot}5$ Not significant
Neo-antergan	2–10 mg./kg.	6	+21·3	± 7·96	$t=3{\cdot}9$, $p<0{\cdot}01$ Highly significant increase
Controls		25	−10·8	± 3·57	

The method shows satisfactorily the inhibition of acid secretion in the stomach produced by recognized inhibitors at suitable dose levels. Neo-antergan shows no inhibition in small doses, and in larger doses the output of acid in the second response is increased.

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The role of calcium in contractures of the rectus abdominis of the frog. By E. J. Denton. *Biophysics Research Unit, University College, London*

Recti abdominis of frog (*Rana temporaria*) were soaked in calcium-free solutions at room temperature for several hours. The long-continued contracture, rising slowly and lasting indefinitely, in response to acetylcholine (10^{-5}) or to potassium (4·5 g. KCl/l., i.e. 30 times that in Ringer), which is typical of the rectus muscle, no longer appeared. The response was now similar to that of the sartorius, rising quickly and lasting less than a minute. Calcium added to the soaking fluid restored the power of the muscle to give long-continued contractures.

The response to acetylcholine or potassium remaining after soaking in calcium-free solution could be abolished by raising the potassium concentration to that required to abolish response to electric shocks, i.e. about 6 times that of Ringer. Curarizing the muscle abolished the response to acetylcholine but not that to potassium.

The addition of calcium which restored the contracture response did not change the threshold or maximum response to alternating current (50 cyc./sec.).

The long-continued contracture to acetylcholine or to potassium increased in amplitude with the calcium concentration up to 0·05 g. $CaCl_2$/l., but was independent of it above this value.

Concerning an excess of sodium and chloride in the aqueous humour of the cat and dog. By H. Davson, W. S. Duke-Elder and D. M. Maurice. *Department of Physiology, University College, London*

If the aqueous humour were a simple dialysate of plasma, sodium and chloride should be distributed between the two fluids in accordance with the Gibbs-Donnan equilibrium. Van Slyke calculated a figure of 1·04 for the ratio of the concentrations of sodium in plasma and its dialysate, and a figure of 0·96 for the corresponding chloride ratio. In the cat and dog the values for the sodium ratio (R_{Na}) have been found to be $1{\cdot}03 \pm 0{\cdot}004$ and $1{\cdot}04 \pm 0{\cdot}003$ respectively (Davson, 1939; Davson & Weld, 1941); these findings suggest that the sodium ion is distributed in accordance with simple electrolyte equilibria. The values of R_{Cl} have been found to be 0·945 and 0·90 for the cat and dog respectively; the ratio for the dog, in particular, is greatly different from Van Slyke's theoretical ratio of 0·96, and it has been suggested that chloride is secreted into the aqueous humour of this animal. Studies with collodion membranes (e.g. by Greene & Power, 1931) indicate that Van Slyke's theoretical ratios, R_{Na} and

R_{Cl}, should be in the region of 1·08 and 0·99 respectively, presumably because the activity coefficients of the ions are not the same in the two fluids. If this is true for aqueous humour and plasma, the two fluids are not in thermodynamic equilibrium with respect to the sodium and chloride ions, the observed ratios being the result of the presence of an excess of these ions in the aqueous humour. On dialysing aqueous humour against plasma, by means of a collodion sac, the excess salt should pass from the aqueous humour into the plasma; as a result, the values of R_{Na} and R_{Cl} should rise; moreover, the conductivity of the plasma should rise at the expense of that of the aqueous humour. Experiments on the aqueous humour of cats and dogs confirm this expectation; the mean value of R_{Cl} in the dog, for example, rises from 0·91 to 0·98 after dialysis, and the mean value of R_{Na} rises from 1·04 to 1·06. In the cat, R_{Na} rises from 1·04 to 1·07 and R_{Cl} from 0·95 to 0·965. With several cats it was observed that R_{Cl} was unchanged on dialysis. Changes in conductivity of aqueous humour and plasma were found, consistent with the migration of salt from the former fluid.

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Electric membrane properties of frog muscle. By BERNHARD KATZ.

Biophysics Research Unit, University College, London

Hodgkin & Rushton (1946) have recently described a method by which the electric constants of non-medullated nerve fibres could be measured. It consists essentially in the application of a rectangular current to the axon surface

TABLE 1. (Temperature range 18·5–28° C., mean 22° C.)

Preparation	Bundles of m. add. magnus (1–4 fibres)	M. ext. longus dig. IV
No. of experiments	9	14
Mean fibre diameter (μ.)	75	43
Characteristic length (mm.)	0·65 (0·47–1·15)	1·1 (0·75–1·5)
Membrane time constant (msec.)	9 (4·6–27)	18·5 (7·3–38)
Specific resistance of myoplasm (ohms cm.)	188 (131–280)	255 (206–355)
Transverse resistance of membrane (ohms cm.^{2})	1500 (650–4500)	4300 (1500–9500)
Membrane capacity (μF./cm.^{2})	6·1* (4·3–10)	4·4† (2·9–5·9)
Percentage external fluid space	17 (10·5–26·5)	27 (17–45)

* S.E. of mean 0·58 μF./cm.^{2} (9 experiments).
† S.E. 0·23 μF./cm.^{2} (14 experiments).

and in an analysis of wave form and attenuation of the potential changes in the extrapolar region. Similar experiments were made on frog muscle using isolated bundles (1–4 fibres) of the m. adductor magnus and the m. extensor longus dig. IV which consists of about fifty fibres. Mean results are shown in Table 1. The value of the membrane capacity is of particular interest, for it is about 5 times greater than the values obtained from various types of non-medullated nerve. The muscle fibre is known to be relatively slow in its electric reactions; the conduction velocity is lower and the time factor of excitation larger than in non-medullated nerve fibres of similar size. It appears that the high electric capacity of the muscle membrane is an important factor in determining these physiological differences.

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The regulation of the afferent discharge from a frog muscle. By B. Katz and J. Z. Young. *Biophysics Research Unit and Anatomy Department, University College, London*

In a recent paper Kuffler & Gerard (1947) have shown that frog muscle is supplied with small motor nerves which elicit slow contractions quite unlike the ordinary muscle twitch. Matthews's (1931) experiments indicate that the intrafusal muscle fibres are innervated by such small axons, and it seemed possible therefore that the contractions described by Kuffler & Gerard were entirely located in the spindles and served to regulate the 'bias' on the stretch receptors. To examine this possibility, the M. ext. longus dig. IV of the frog was isolated together with its nerve, and recording electrodes were placed on nerve and muscle so as to obtain a simultaneous picture of electric muscle responses and afferent discharge. In some experiments the nerve supply was reduced by dissection until one or two sensory and one or two motor units remained.

(i) With stimulation of the low threshold motor axons, afferent impulses arise as early as 8 msec. after the muscle spike. This discharge is frequently still obtained during an intermediate stage of neuro-muscular block, when all perceptible motor responses have vanished. Thus the sense organ appears to be triggered by an extremely delicate mechanism which can be activated via branches of the ordinary motor axons. (ii) The occurrence of local responses of the muscle to stimulation of high-threshold fibres (Kuffler & Gerard, 1947) was readily confirmed. They are sometimes, but not invariably, followed by a sensory discharge. The fact that these local contractions can be obtained *without* change of the afferent discharge seems to eliminate the possibility that they are an integral part of the spindle mechanism and lends support to the suggestion (see Kuffler & Gerard, 1947) that they serve some separate motor function.

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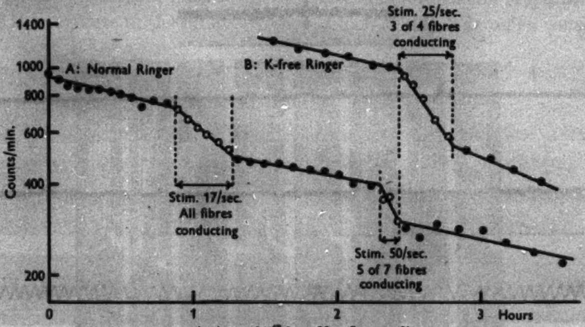
The leakage of radioactive potassium from stimulated nerve. By R. D. Keynes. *George Henry Lewes Student, Physiological Laboratory, Cambridge*

Carcinus nerves were soaked for several hours in Ringer containing K^{42}, until they had taken up an appreciable amount of the radioactive ions. The nerve was then mounted in a thin-bottomed chamber over a Geiger counter, with its ends pulled up into a layer of paraffin oil so that stimuli could be applied and the action potentials recorded. The central part of the nerve was held down by glass hooks, and inactive Ringer was circulated continuously around it so that K^{42} ions did not contribute to the recorded radioactivity once they had leaked out. Under resting conditions the amount of K^{42} in the fibres then decreased exponentially, with an average half-time of 1·4 hr. for whole nerve, and 1·9 hr. for isolated 30 μ. fibres.



Leakage of K^{42} from 30μ. *Carcinus* fibres

In the figure above it is seen that when the nerve was stimulated at 17 impulses/sec., the rate of loss of K^{42} was roughly tripled. In normal Ringer this might have been due either to an increased rate of exchange of K^+ ions, with no net loss of K, or to an actual leakage of K during stimulation. The fact that the same increased loss of K^{42} occurred in K-free Ringer, where there could be no exchange, suggested that there was a net leakage of K. This was confirmed by comparing the rates of entry of K^{42} into resting and stimulated fibres; at 50 impulses/sec. the rate was only increased to 1·3 times its resting value, whereas the corresponding increase in rate of leakage would be about 10 times.

The total amount of K inside the fibres which could exchange with K^{42} was found to be 206 mmol./l. axoplasm. Hence the amount of K lost during stimulation was calculated to be $2 \cdot 1 \times 10^{-12}$ mol./cm.^{2} membrane/impulse. This is comparable with the figure of $1 \cdot 7 \times 10^{-12}$ mol./cm.^{2}/impulse obtained by Hodgkin & Huxley (1947) for the same type of nerve by an indirect method, and supports the supposition that K does leak out during activity.

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Slowly adapting touch receptors in skin from the rabbit's leg.

By B. Frankenhaeuser (introduced by G. Weddell). *Department of Human Anatomy, University of Oxford*

An investigation has been made of the action potentials aroused in the sural (saphenous minor) nerve in the rabbit following natural stimulation of the skin in its distribution. So far it has been found that two different types of response can be recorded. The first of these has already been described by Adrian, and is derived from rapidly adapting hair touch receptors. The second, however, does not appear to have been previously noted, and consists of action potentials from receptors with a slow adaptation. Such potentials can be elicited by the stimulation of some four or five points per square centimetre of skin. This type of response cannot be aroused by bending the hairs, but only by stimulating

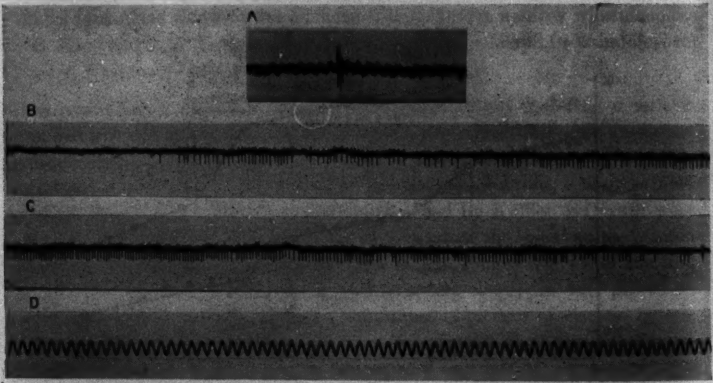


Fig. A. Action potentials from the sural nerve produced by bending a few hairs with a No. 1 nylon thread, and keeping the hairs in this position.

Figs. B, C. Record obtained when a slowly adapting receptor was touched with a No. 1 nylon thread. The increase in frequency followed an increase in pressure. B and C are continuous.

Fig. D. Time marking 50 cyc./sec.

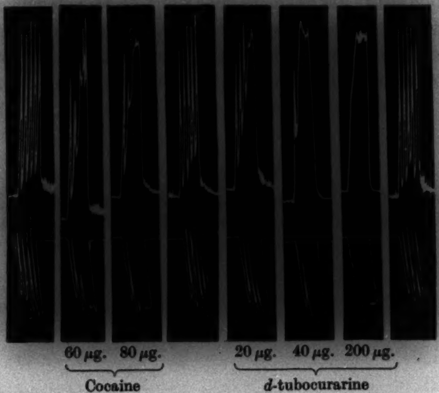
the actual skin surface, and if the skin is moved on the deeper tissues the points concerned are found to move with it. These receptors may be stimulated with a blunt rod, but their threshold is even less than the pressure of a No. 1 nylon thread (200 mg.; 0·15 mm. diameter). The discharge frequency is higher if higher pressures are used; the response stops as soon as the pressure or touch on the skin is removed.

The neurohistology of the skin in the points concerned in this response is under active investigation, but it is too soon to report results, except to note that Pacinian corpuscles do not appear to be responsible for this effect.

It is also of interest that if the nerve is blocked by means of a pressure cuff round the intact leg, the fibres conducting the action potentials from hair touch receptors are blocked five to ten minutes before the fibres conducting impulses from the slowly adapting receptors.

Inhibition of the peristaltic reflex as a method for assaying local anaesthetics and curare-like substances. By W. Feldberg* and C. Y. Lin† (Chengtu). *From the Physiological Laboratory, Cambridge*

The peristaltic reflex was initiated in the isolated rabbit's ileum preparation by raising the pressure in the lumen (Trendelenburg, 1917). The reflex consists of a contraction of the longitudinal muscle, which is a muscular response to the stretching, and of contractions of the circular muscle initiated by a local nerve



Rabbit's ileum preparation in 40 c.c. Tyrode solution. Upwards stroke of upper tracing shortening, lower tracing volume record of intestine. Time 30 sec.

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* With a grant from the Medical Research Council.

† With a British Council Fellowship.

reflex. The contractions of the circular muscle, which are easily recorded with a volume recorder, are inhibited by local anaesthetics and curarine in doses too weak to affect the muscle directly. The inhibition is reversible and repeatable, and with increased doses graded responses are obtained. Inhibition of the reflex thus provides a simple method for testing the activity of these drugs. In a 40 cm. bath the threshold dose for *d*-tubocurarine was about 10 μg., for cocaine about 20 μg. Procaine was slightly less, borocaine 7 times less and nupercaine was about 12 times more active than cocaine; in addition, the effect of nupercaine lasted longer than that of cocaine after removal of the drugs.

The usual procedure adopted was to initiate the reflex every fourth minute by raising the pressure in the lumen to about 3 cm. saline and keeping it high for 1 min. The drugs were added 2 min. before, and washed out when the pressure was again lowered to zero. Between each test two normal control periods of peristalsis were allowed; with one exception they are omitted in the figure, which shows the initiation of the reflex and its graded inhibition by different doses of cocaine and *d*-tubocurarine. The contraction of the longitudinal muscle, however, is not inhibited.

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PROCEEDINGS
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PHYSIOLOGICAL SOCIETY

10 *April* 1948

The effect of high and low plane feeding on the serum protein levels of pregnant ewes and foetuses.

By A. DALGARNO, W. GODDEN and E. F. MCCARTHY

Two groups of pregnant ewes were fed at different dietary levels, one on a high-plane diet maintaining or increasing maternal weight, and the other on a low-plane diet resulting in a loss of maternal weight. The serum protein of foetuses, lambs and ewes was estimated from Kjeldahl nitrogen values, and A/G ratios were determined by magnesium sulphate precipitation and electrophoresis. The serum protein level in the foetus was found to be constantly lower than that of the adult sheep. High-plane fed mothers and foetuses have a higher serum protein level than low-plane animals. The ewe serum albumen tends to rise and the electrophoretically slowest globulin component to fall at the end of pregnancy. The latter component is either absent or present in small amount in the sera of newborn lambs and appears after colostrum feeding (cf. Charlwood & Thompson, 1948). A spike resembling the anomaly described by Longsworth, Shedlovsky & MacInnes (1939) appears in the electrophoretic pattern of the 10-day old lamb serum.

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PROCEEDINGS
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22 *May* 1948

Changes in transparency of muscle during a twitch. By D. K. Hill.

Physiological Laboratory, University of Cambridge

When the sartorius muscle of a frog is stimulated the action potential rises to its peak in 2 msec. at 18° C. The first sign of mechanical activity is a transient lengthening (the 'latency relaxation'; Sandow, 1944) which starts about at the moment when the action potential reaches its peak and reverses into a positive contraction a few msec. later. Transparency changes have not hitherto been recorded with an amplification sufficient to determine the exact moment at which they start (Schaefer & Göpfert, 1937).

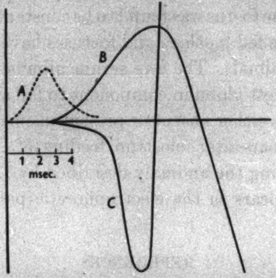

Fig. 1. *A*, action potential. *B*, transparency record. The positive direction denotes an increase. *C*, mechanical record. The negative direction denotes a decrease in tension. Temperature 18° C.

The apparatus to be demonstrated is for recording the transparency of a frog's sartorius muscle during a twitch. The earliest change, an increase in transparency, is found to start at the same time as the latency relaxation: the latter is recorded with a crystal pick-up. The effect is present at all wavelengths in the visible spectrum. This first phase of the transparency change, during the latency relaxation, is of a different nature from the larger change which follows it. It will be shown how the two phases can be distinguished from one another.

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The action of botulinum toxin on the isolated rat diaphragm.

By A. S. V. Burgen, F. Dickens and L. J. Zatman. *Department of Pharmacology and the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School*

If type A botulinum toxin in amounts of 1000–5000 mouse LD_{50}/ml. ($\equiv$0·03–0·15 μg. pure toxin/ml.) is added to the Tyrode in which a rat phrenic nerve-diaphragm preparation (Bülbring, 1946) is immersed, no change in response to nerve stimulation is seen for about 20–30 min., but a rapid paralysis then ensues during the next hour (Fig. 1). Conduction in the nerve trunk is unaffected and the muscle remains normally responsive to direct stimulation. Heating the toxin to 100° C. for 15 min. destroys all activity. The paralysis is not reversed by replacing with fresh Tyrode, and, indeed, washing the toxin out even during the latent period has no effect on the ultimate paralysis unless this is carried out during the first few minutes. The latent period and course of paralysis are greatly prolonged by low temperatures. The neuromuscular block produced by toxin differs from that produced by curare in that motor-nerve tetani are well maintained, the summation curve for two stimuli is normal in type, and there is no antagonism by eserine, prostigmine, tetraethylpyrophosphate, KCl or adrenaline. The muscle remains normally sensitive to close intravascular acetylcholine, but the output of acetylcholine from the preparation during a motor-nerve tetanus is greatly reduced. The enzyme systems acetylating choline and sulphanilamide, and also 'true' and 'pseudo' cholinesterases, are unaffected by the toxin. Antibotulinum serum will prevent the toxin acting, but will not restore the partly paralysed preparation. Diaphragms from rats made actively immune with botulinum toxoid are highly resistant to the action of the toxin.

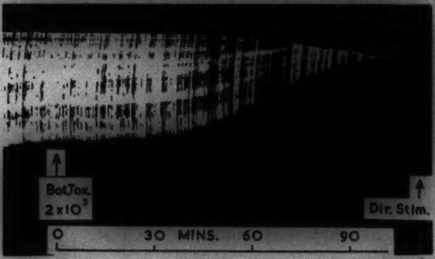

Fig. 1. Rat phrenic nerve-diaphragm preparation. Stimulation 6/min. At arrow 2000 mouse LD_{50} toxin/ml. added to bath. Direct stimulation gives a normal response.

We are indebted to Dr D. W. Henderson for supplies of toxin and to the Chief Scientist, Ministry of Supply, for permission to make this publication.

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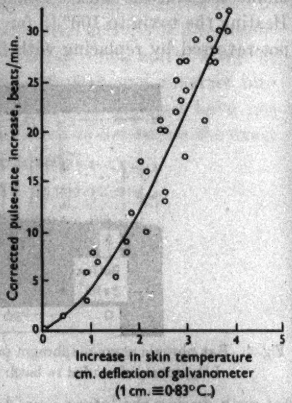
Changes in pulse rate during exposure of the skin to radiant energy.

By K. E. Cooper and D. McK. Kerslake (introduced by A. St G. Huggett). *Physiology Department, St Mary's Hospital Medical School*

Healthy young male subjects were exposed to radiation from six 100 W. electric-light bulbs, arranged inside a reflecting metal heat cradle. The head and legs were protected from draughts by thin blankets.

The mouth temperature fell about 0·3° C., returning to its previous value after about 25 min., and continued to rise thereafter. The blood pressure showed no appreciable variation during the apyrexial phase. The pulse rate rose rapidly at first and then more slowly, the rise commencing during the first minute. The form and magnitude of this increase was remarkably constant on different occasions for any one subject.

Thermocouples were inserted into the skin of the chest and their temperatures taken as an index of the mean temperature of the exposed skin. An attempt was made to confirm the supposition of Benson (1934) that there is a relation at any moment between the rise of skin temperature and the increase in pulse rate from their resting levels. At various rates of heating there appeared to be no correlation.



The relation between the adjusted heart rate increase and the rise in skin temperature.

Lyon (1926) has shown by a statistical analysis that in man a change in body temperature of 1° F. alters the heart rate by about ten beats per minute. When the observed pulse-rate increase was adjusted on this basis, allowing thereby for the fall in mouth temperature, a relationship was found to exist between the rise in skin temperature and the rise in heart rate.

We have some evidence which suggests that halving the area of skin exposed halves the response obtained.

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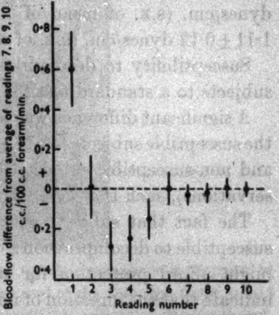
Changes in forearm blood flow following the application of an arterial occlusion cuff to the wrist. By D. McK. Kerslake. *R.A.F. Institute of Aviation Medicine, Farnborough*

Blood flow in the left forearm was measured by the venous occlusion plethysmograph. The collecting pressure was applied for periods of 5 sec., during which records of forearm volume changes were obtained. The interval between consecutive applications of the collecting pressure was 10 sec., and the cycle was maintained throughout the whole experimental period.

The subjects, who were healthy young men, were seated at rest in a room at about 20° C. for a quarter of an hour before the experiments were begun. A sphygmomanometer cuff fitted round the left wrist was inflated to 230 mm. Hg from a reservoir, and following this ten records were taken of the forearm blood flow, the first starting 5 sec. after the application of the wrist cuff. The wrist cuff was then released and recording was stopped. The procedure was repeated at intervals of 5 min. till ten such runs had been recorded.

Individual runs showed no consistent pattern, but the readings after the first six—i.e. after the first minute—tended to stabilize. There were appreciable differences between the levels of blood flow for the same subject during different runs. To leave out of account secular fluctuations of this kind, the deviations of the readings from the mean of the last four were calculated for each run. The ten sets of results for the subject were averaged.



Changes in forearm blood flow after application of a high pressure cuff round the wrist. Mean values for five subjects. Vertical lines represent 95 % confidence limits of means.

The curves obtained in this way for five out of six subjects did not differ significantly. The sixth subject showed the same general changes but with different time relations. The results from the five similar subjects were pooled, and from these data confidence limits for the means were obtained, demonstrating the existence of a typical fluctuation in forearm blood flow during the first minute after the application of the wrist cuff.

Serum surface tension and its relation to the decompression sickness of aviators. By D. N. Walder. *Department of Physiology, The University, Bristol*

Theoretically the formation, stability, rate of growth and ultimate size of gas bubbles in a fluid is affected by the surface tension of the fluid. A low-surface tension favours all aspects of bubble formation and development.

On the assumption that the decompression sickness of aviators is due to the occurrence of intravascular bubbles (Catchpole & Gersh, 1947), an investigation was carried out on the surface tension of the circulating fluids in man and its possible relationship to the susceptibility to decompression sickness.

Serum was used for the determinations to avoid errors due to the presence of anticoagulants.

The static surface tension was measured at 25° C. by the ring and torsion balance method (du Nouy, 1926). The range of static surface tension of human serum was found to be 48·10–53·80 dynes/cm. with a mean of $50{\cdot}22 \pm 0{\cdot}13$ (S.E. of mean of 97 observations). There was a diurnal variation of $0{\cdot}65 \pm 0{\cdot}09$ dynes/cm. (S.E. of mean of 7 observations) and a day to day variation of $1{\cdot}11 \pm 0{\cdot}12$ dynes/cm. (S.E. of mean of 18 observations).

Susceptibility to decompression sickness was determined by exposing the subjects to a standard test (37,000 ft. for 2 hr.).

A significant difference was found between the mean static surface tension of the susceptible subjects ($49{\cdot}14 \pm 0{\cdot}13$ dynes/cm.; S.E. of mean of 38 observations) and non-susceptible subjects ($51{\cdot}72 \pm 0{\cdot}22$ dynes/cm.; S.E. of mean of 59 observations), such that $t = 18{\cdot}54$ and $P = < 0{\cdot}001$.

The fact that subjects with a high static surface tension tend to be less susceptible to decompression sickness suggested that raising the surface tension might afford protection for susceptible subjects. Preliminary experiments indicate that the ingestion of normal saline by susceptible subjects immediately prior to a test is followed by a temporary rise in static surface tension and freedom from decompression sickness.

This work was carried out at the R.A.F. Institute of Aviation Medicine, Farnborough, and permission to publish these results has been given by the Air Ministry.

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The phrenic nerve-diaphragm preparation of different species in the estimation of curare-like activity. By R. Wien. *Biological Division, May and Baker Ltd., Dagenham*

In a study of the curare-like activity of certain synthetic substances we found that a compound, R.P. 3697, the triethyliodide of tri(diethylaminoethoxy)-1-2-3 benzene, described by Bovet (1947), was about one-third as active as *d*-tubocurarine when assayed by the rabbit head drop method or on the sciatic-gastrocnemius preparation in the cat. Its effects were easily reversible by prostigmine. However, when assayed by the rat phrenic nerve-diaphragm preparation (Bülbring, 1946; Chou, 1947), it was only about 1/100 as active as *d*-tubocurarine. We therefore tried the isolated phrenic nerve-diaphragm preparations of other species, and found that preparations made from very young rabbits or kittens (body weight about 150 g.) worked quite satisfactorily.

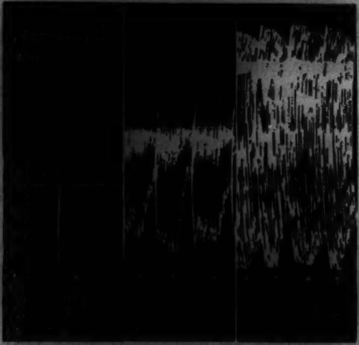

Fig. 1. Phrenic nerve-diaphragm preparations. Record of muscle contractions in response to maximal stimulation of single shocks 8 per min. (The speed of the drum was faster in (*a*).) *C*=*d*-tubocurarine chloride; *R.P.*=R.P. 3697, the triethyliodide of tri(diethylaminoethoxy)-1-2-3 benzene. Magnification ×½.

The assays were carried out at 20° and KCl (40 mg. to an 80 c.c. bath) was added during the recovery phase (West, 1947). Doses of the compound were added at intervals of 8–12 min., and the slow decline of the muscle twitches in the kitten's diaphragm (Bülbring, 1946) was not observed. Similar results were obtained at 37° and when the rate of stimulation was increased to fifteen shocks per minute.

It will be observed from Fig. 1 that in both the rabbit and kitten preparations the synthetic compound was equal to, or one-fifth as active as, *d*-tubocurarine, but on the rat diaphragm there was a marked loss in activity. All three preparations appeared to be equally sensitive to *d*-tubocurarine itself.

I am indebted to Mr G. T. Langston for valuable assistance.

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Carbohydrate feeding and the Houssay cat. By A. H. Bone and C. Reid. *Department of Physiology, London Hospital Medical College*

It has been stated that insulin in in vitro experiments opposes the inhibitory action of pituitary extracts on the hexokinase reaction. If this holds for the normal organism, then the carbohydrate metabolism of the Houssay (chronically hypophysectomized-depancreatized) cat should be normal. To test this hypothesis, measurements of the respiratory exchanges were made in fasting (18 hr.) normal, hypophysectomized and Houssay cats. Following 30 g. glucose per $m.^2$ body surface by stomach tube, the R.Q. of normal and hypophysectomized cats increased from about 0·74 to over 0·9, whereas the change in the Houssay cat was small, e.g. from 0·7 ± 0·02 to 0·74 ± 0·02. Moreover, the blood-sugar rise in the Houssay cat after glucose was greater and more prolonged than for the normal cat.

Further, the R.Q. of the Houssay cat given glucose and adequate insulin rose nearly as much as in the normal cat given glucose alone. This showed that the tissues of the Houssay animals were still capable of metabolizing sugar.

It appears, therefore, that carbohydrate metabolism in the Houssay animal is not normal.

The action of phosphates on neuromuscular transmission in the frog. By G. L. Brown and M. Vianna Dias. *National Institute for Medical Research, London, N.W. 3, and Instituto de Biofísica, Rio de Janeiro*

Brazilian frogs (*Leptodactylus ocellatus*), 80–150 g. in weight, were decapitated and the gastrocnemius muscle was prepared for arterial injections into the tibial artery (cf. Brown, 1937). The muscle of this frog is less sensitive to acetylcholine than is the gastrocnemius of *Rana esculenta*. Repeated injection into the muscle of Ringer's solution containing 0·5% NaH_2PO_4 produces neuromuscular block. Perfusion of the muscle with 0·2% NaH_2PO_4 in Ringer's solution produces a similar neuromuscular block which is reversible. When transmission from nerve to muscle has ceased, the muscle retains its sensitivity to acetylcholine. The block, therefore, differs from that produced by curarine in which the response to acetylcholine is lost before that to nerve stimulation. The block is probably due to failure of acetylcholine production at the motor nerve endings, and is similar to that caused by Ca^{++} deficiency.

Substances producing neuromuscular block should not be classified as 'curarizing substances' until their effect on the sensitivity of the muscle to acetylcholine has been determined.

A full account of this work has been published in *An. Acad. Brasileira Ciências*, 1947, **19**, 359.

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Independent light-detectors in the peripheral retina.

By M. H. PIRENNE.* *Department of Physiology, University College, London*

Many observations suggest that the peripheral retina is a mosaic of units, each 2° diameter or so, which, *under near-threshold conditions of stimulation*, behave as independent light-detectors (Pirenne, 1945). Thus, when dim light is flashed over a wide field, only a few detectors among many might be stimulated. Yet such a field has not the appearance of a pattern of discrete spots. Introspection, however, may be unreliable. Experiments were made to test the above theory.

The following stimuli are presented to a dark-adapted subject, in an unknown sequence: a circle, 20° in diameter, having its centre 20° from a fixation point (○); the upper half of this circle (◠); its lower half (◡). Using dim, constant, flashes (0·03 sec.), the area of these fields can be uniformly illuminated. The subject knows the conditions of the experiment. When he has seen the light, he reports which of the stimuli he thinks was used. Typical results were as follows:

Stimulus ○. Reported correctly as 'circle' 7/20 (i.e. in 7 out of 20 trials). Incorrectly: as 'upper half' 1/20; as 'lower half' 2/20. Not seen 10/20.

◠ Correctly 1/20. Incorrectly: as 'circle' 4/20; as 'lower half' 1/20. Not seen 14/20.

◡ Correctly 2/20. Incorrectly: as 'circle' 3/20. Not seen 15/20.

The many wrong answers given show that confusion occurs between full and half circles. This must be expected theoretically. If a few detectors only are stimulated in a circle, they sometimes will be all, say, in one given half of the circle. Then the stimulation by the whole circle will be indistinguishable from stimulation produced by this half only. On the basis of the independence of the detectors, this reasoning may be pursued quantitatively.

The chance of seeing nothing when the stimulus is ○ is equal to the product of the corresponding chances for ◠ and ◡, i.e. $(14/20) \times (15/20) = 0{\cdot}52$. This is close to the observed value, 10/20.

The chance that the retina be stimulated by *only* the upper half of ○ is the chance of seeing ◠ at all, multiplied by that of *not* seeing ◡. Since the subject reports ◠ correctly in only 1 out of 6 trials in which light was seen, the chance of ○ being *reported* as 'upper half' finally is $(1/6) \times (1 - 14/20) \times (15/20) = 0{\cdot}038$, as against 1/20, the observed value. Corresponding results for ○ reported as 'lower half' are 0·07 against 2/20.

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* Imperial Chemical Industries Research Fellow of the University of London.

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Pressor responses caused by electrical stimulation of the vagus and sinus nerves in the cat anaesthetized with nembutal. By W. W. Douglas, I. R. Innes and H. W. Kosterlitz. *Department of Physiology, University of Aberdeen*

In the cat anaesthetized with nembutal (intraperitoneal, 40 mg./kg. body weight) and with both vagi cut, the right and left sinus nerves and the central ends of the right and left vagus were stimulated by a neon lamp stimulator (Whitfield, 1946) and depressor vasomotor responses obtained. While employing the same stimulus the fall of blood pressure was usually converted to a rise on the addition of further amounts of nembutal. The pressor responses occurred in some animals after the addition of half the anaesthetic dose. These pressor responses to stimulation of the vagus and sinus nerves have been found to be independent of the lowered level of blood pressure at which they usually occur, and they persist on removal of the cerebral hemispheres, the pituitary gland, cerebellum and all of the brain stem above the auditory tubercles.

The preparation may show spontaneous reversion of the pressor to the depressor response after some time. Intravenous injection of picrotoxin in animals with the central nervous system intact, and also in those with all brain matter above the level of entry of the 8th nerves removed, rapidly converts the pressor response into a depressor one. Nembutal given intravenously at this stage may cause the reappearance of the pressor response.

In order to exclude the possible effects of altered pulmonary ventilation on the blood pressure, many of the experiments have been conducted under artificial respiration with bilateral open pneumothorax.

This phenomenon may be related to that reported by Neil, Redwood & Schweitzer (1948), who found that intravenous injection of chloralose in the decerebrate cat or in cats under nembutal anaesthesia converted the depressor response obtained by electrical stimulation of the sinus nerve to a marked pressor response.

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Abdomino-testicular temperature gradients. By R. G. Harrison and J. S. Weiner. *Climatic Unit, Department of Human Anatomy, Oxford*

Testicular descent is generally thought to ensure a temperature below abdominal presumably to favour normal spermatogenesis (Badenoch, 1945). This author found an abdomino-scrotal difference av. 2·2° C. in thirty adult herniorraphies at temperatures of 70–78° F. Harrenstein (1928) noted a somewhat greater difference in children. We have recently measured simultaneously testicular and abdominal temperatures in various animals, since comparative data are surprisingly scanty.

The copper-constantan (both s.w.g. 46) thermocouple in a fine plastic tube with tip bare was inserted by fine hypodermic needle tubing which was then withdrawn. The couple was drawn back about 2 mm. at a time so as to obtain the maximum testicular temperature. Readings were taken quickly after anaesthesia as abdominal temperatures are apt to fall quickly. Just where to take scrotal temperatures is rather uncertain. Still air conditions prevailed.

Animal	Date	Abd. temp. (° C.)	Testis temp. (° C.)	Abd. testis diff. (° C.)	Abd. scrotal diff. (° C.)	Room temp. (° F.)
Guinea-pig	5. i. 48	39·3	35·2	4·1	5·6	65
	6. i. 48	39·5	35·5	4·0	4·5	64
Rat	5. i. 48	39·7	31·0	8·7	9·5	65
		37·7	29·8	7·9	9·3	65
Mouse	5. i. 48	38·2	29·8	8·4	9·7	65
	9. i. 48	37·1	28·5	8·6	11·2	62
Rabbit	1. i. 48	38·9	32·4	6·5	7·0	66
	5. i. 48	39·8	33·6	6·2	6·5	65
	2. ii. 48	39·5	33·6	5·9	6·2	65
Ram	25. ix. 47	42·1	34·9	7·2	13·0	64
	20. x. 47	39·7	32·6	7·1	13·0	66
Goat	20. i. 48	38·4	32·9	5·5	8·3	64
	7. iv. 48	38·4	33·7	4·7	7·9	68
Dog	5. xii. 47	36·6	34·1	2·5	4·1	67
Rhesus	11. xi. 47	36·6	34·8	1·8	3·7	75
	1. iv. 48	35·5	33·2	2·3	2·9	64

Despite the lability of the temperatures in these conditions, the abdomino-testicular and also abdomino-scrotal gradients are clearly much bigger in some animals than others. The abdomino-scrotal figures for guinea-pig and rat but not rabbit are of the order given by Moore & Quick (1924).

Many factors are obviously involved in producing the abdomino-testicular temperature gradient—size, shape, position, coverings (Crew, 1921) and intrinsic heat production of the testis. Features not apparently hitherto taken into account concern the testicular blood flow, for the tortuosity of the testicular artery and its relation to the pampiniform plexus would appear to provide a likely mechanism for ensuring this temperature gradient. Remarkable differences in vascular pattern can also be demonstrated in the animals listed. Further anatomical and physiological data will be reported elsewhere.

We are indebted to Prof. H. C. Bazett for the method of soldering the couples and also for the gift of plastic tubing.

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X-ray investigation of transparent oil-water systems. By J. H. SCHULMAN, T. S. MCROBERTS and D. P. RILEY. *University of Cambridge*

It has been shown by Hoar & Schulman (1943) and Schulman & McRoberts (1946) that equal volumes of oil and water can be dispersed in one another by soap and various alcohols, to give electrically non-conducting or conducting systems which are transparent and of low viscosity.

It was suggested that these systems consisted of uniform close-packed water or oil droplets, whose diameters were, in the concentrated soap solutions, of molecular dimensions. These droplets could be made to swell gradually by decreasing the soap and alcohol concentrations to give Tyndall and other optical scattering effects. The oil or water droplets are considered to be covered by an equimolecular mixed film of soap and alcohol (polar oil) molecules and the wettability of this mixed monolayer by the oil or water determines the continuous phase. Thus with the system water-potassium oleate-*p*-methyl cyclohexanol-benzene, water droplets are dispersed in the benzene, but with the system water-potassium oleate-*p*-methyl cyclohexanol-paraffin (Thin Nujol), paraffin droplets are dispersed in the water continuous phase (Schulman & McRoberts, 1946) (see Fig. 1).

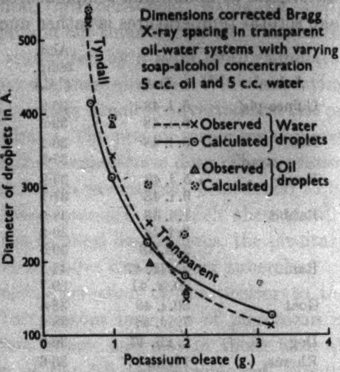

Fig. 1.

The dimensions obtained from low-angle X-ray bands agreed with the calculated dimensions, based on the conception of uniform water spheres in oil or uniform oil spheres in water (see Fig. 1). The diameters of the droplets obeyed the formula

$$\frac{3 \times \text{volume dispersed phase}}{\text{area interfacial monolayer}} = \text{radius of oil or water droplet} = r_1.$$

The packing of the soap-alcohol molecules was deduced from monolayer penetration experiments (Schulman & McRoberts, 1946), assuming an area per pair of soap-alcohol molecules of 60 A.^{2} and a thickness of the interfacial monolayer of 24 A. The water droplets give stronger and less diffuse X-ray scattering than the oil droplets. Diameters of the droplets up to 600 A. have so far been measured, the larger ones giving a strong Tyndall scattering effect. It is hoped in this way to bridge the gap between swollen micelles and emulsions and to correlate X-ray and microscopic observations on the structure of colloidal solutions.

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